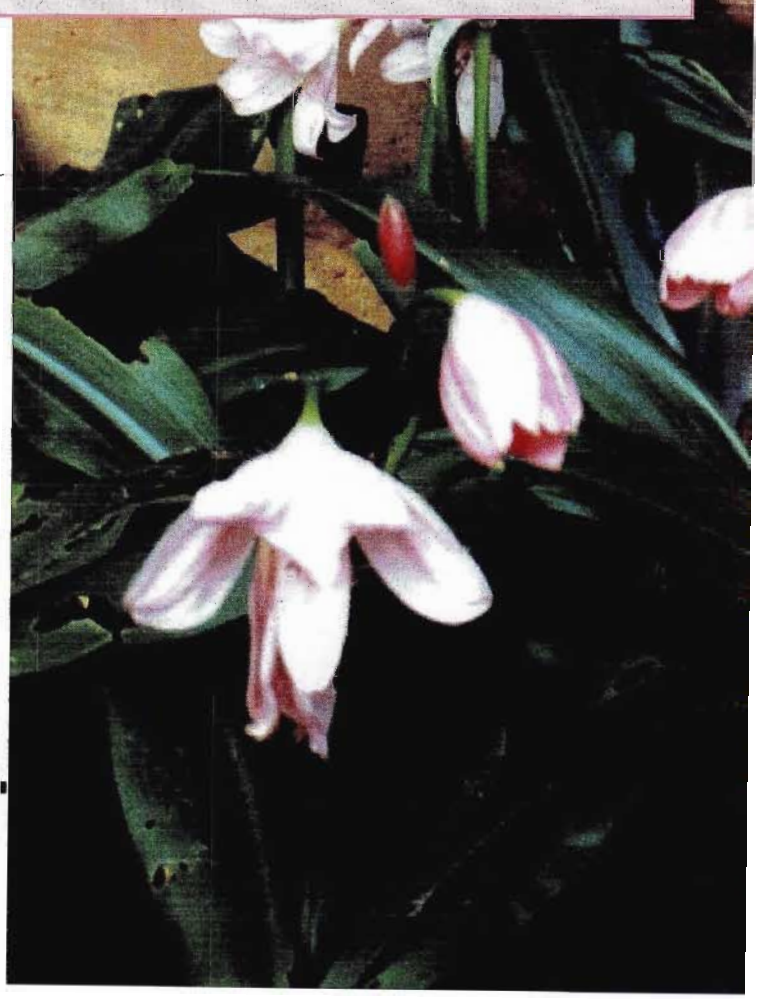
Crinum moorei

Propagation and secondary metabolite production *in vitro*



Submitted in fulfilment of the
requirements for the degree of
DOCTOR OF PHILOSOPHY

CATHERINE FENNELL

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

2002

Crinum moorei: Propagation and secondary metabolite production *in vitro*

table of contents

Declaration

I hereby declare that this thesis, unless acknowledged to the contrary in the text, is the result of my own investigation under the supervision of Professor J. van Staden, Research Centre for Plant Growth and Development, School of Botany and Zoology, University of Natal, Pietermaritzburg.

C. Fennell

Catherine W. Fennell
April 2002

I certify that the above statement is correct.

J. Staden

Professor J. van Staden
(Supervisor)
April 2002

Abstract

As an alternative to conventional methods of vegetative propagation, micropropagation attracts much attention, because the levels of multiplication are increased, somaclonal variation is limited and disease-free material can be obtained. The technique is invaluable to the conservation of *Crinum* species belonging to the Amaryllidaceae which, as a group, possesses several biological features that make them particularly vulnerable. This is in addition to other problems relating to their value as horticultural material, traditional medicines and sources of phytochemicals of interest to medical science.

Two *in vitro* systems are widely used for the propagation of amaryllidaceous species; regeneration from young floral stem explants and from twin-scales excised from bulbs. Although plantlet regeneration could be obtained from peduncle explants of *Crinum moorei*, a complex of factors including: the age of the floral stem; explant position and; hormonal factors, limited growth. Callus production was poor and indirect organogenesis could not be achieved.

Twin-scales were used for the induction of somatic embryos. Morphologically these were different depending on the concentrations of 2,4-D and BA used in the induction medium. Although some of them went on to germinate, the use of somatic embryos for large-scale culture is not an efficient micropropagation route, owing to the low frequency of both embryo production and germination and to the long culture times.

Regeneration of shoots and bulblets could, however, be readily induced from twin-scales using a series of modified MS media, and this despite the fact that explants from the bulb were more difficult to decontaminate than the above ground parts. Shoots arose in the axes of the twin-scales close to the basal plate. Initiation was greatest on a basic Murashige and Skoog medium, containing 4 g ℓ^{-1} sucrose, and in the dark. No hormones were required. At high concentrations, the hormones stimulated abnormal organogenesis. Bulbing of the shoots was further enhanced using higher than normal levels of sucrose i.e. 6% and 5 g ℓ^{-1} activated charcoal. The response was also influenced by the size of the twin-scale and its position in the parent bulb. Greater numbers of bulblets with larger diameters developed in large twin-scales from an intermediate position between the inner and outer scales. Furthermore, light, and a temperature of 25°C were required for normal bulblet development.

Bulblets grown in this manner were used as a source of secondary explants by splitting them vertically in half. The addition of 10 mg ℓ^{-1} BA resulted in multiple shoot development. In a

liquid-shake culture system, this same multiplication medium induced the formation of meristemoid clusters whose rates of proliferation were higher than that achieved for shoot multiplication on either solid or static liquid media. The advantage of using meristematic clusters is that shoot hyperhydricity is avoided. Furthermore, the clusters can be mechanically separated; making the system ideal for automated plant production. Shoot morphogenesis, followed by the formation of bulblets occurred on solid MS media containing activated charcoal or high concentrations (6%) of sucrose. The induction of bulblets by sucrose was, however, slower, which may be beneficial for long-term storage and conservation *ex situ*. Compared to smaller bulblets, bulblets with a diameter of approximately 9 mm, acclimatized readily and grew rapidly after transferring them to the soil in greenhouse conditions.

Biotechnological processes such as cell and tissue culture provide an ideal system for producing secondary metabolites, especially where their production *in situ* is hampered by poor resource availability or when chemical synthesis is difficult. *In vitro* produced *Crinum moorei* bulblets were found to contain nine alkaloids of the Amaryllidaceae type; three of which were released into the culture medium. Light was essential for alkaloid biosynthesis while the inclusion of BA and activated charcoal stimulated the production of specific alkaloids.

Acknowledgements

The following people and organizations contributed to this project and I am greatly indebted to them for their efforts. Thank you to:

Professor J. Van Staden - as supervisor - for allowing me to pursue my own interest in the tissue culture of crinums; for your patient support; guidance and comments during the preparation of this manuscript.

The University of Natal for their financial support in the form of an annual research grant, staff bursary and furlough leave to complete this work.

My Family for sharing my interest in this project; their assistance in collecting specimens and above all, their patience in so many instances.

The staff and students working in the tissue culture laboratory for demonstrating techniques; their helpful advice based on a collective wealth of experience and, in general, in sharing in the day to day running of the laboratory. I am particularly grateful to Dr J. Finnie, Dr N. Jones, Mrs B. Nixon (nee McAlister) and Dr S. McCartan.

Dr E. E. Elgorashi for assisting with the extraction and analysis of *Crinum* alkaloids.

Other academic, technical and support staff for helping to maintain the collection of plants in the garden and greenhouses (Mr C. Hills); for adjusting conditions in specific growth chambers necessary for growth-limiting experiments (Mr M. Hampton); for ordering general supplies (Mr R. Noble); for advice with respect to photographic work (Mr R. Roth); for ordering reprints (The staff of the Life Sciences Library) and assistance with statistical analysis (Professor R. Beckett).

Mr B. Tarr of the National Botanical Gardens, Pietermaritzburg, for supplying some of the plant material.

To all I extend my sincere thanks.

List of abbreviations

ABA	abscisic acid
ANC	ancymidol
ANK	modified Murashige & Skoog medium + ancymidol, NAA and kinetin (SLABBERT *et al.*, 1993)
ANOVA	analysis of variance
β	Greek symbol for beta
B	boron
BA	benzyladenine
Benomyl / Benlate™	[methyl-(butylcarbamoyl)-2-benzimidazole-carbamate]; commercially available as benlate
C	celsius
Ca^{2+}	calcium ions
CM	coconut milk
Cu and Cu^{2+}	copper and copper ions
cv	cultivar
de novo	"from the beginning, anew"; arising, sometimes spontaneously, from unknown or very simple precursors
DW	dry weight
et al.	*Et alia* - "and others"
EU	European Union
ex vitro / extra vitrum	"from glass"; organisms removed from culture and transplanted, generally to soil or potting mixture
Fe and Fe^{2+}	iron and iron ions
FW	fresh weight
GA (including GA_3)	gibberellic acid
G.C. or GC	gas chromatograph
G.I. or GI	growth index
g ℓ^{-1}	grams per litre
H^+	hydrogen ions
HAEM	haemanthamine

$HC\ell$	hydrochloric acid
$HgC\ell_2$	mercuric chloride
HIV	human immunodeficiency virus
$HOC\ell$	hypochlorous acid
HWT	hot water therapy / treatment
IAA	indole acetic acid
IBA	indole butyric acid
i.d.	internal diameter
IEDCs	induced embryogenic determined cells
in vitro	"in glass" i.e. in a test tube
in vivo	biological process that occurs within living organisms
IUCN	International Union for Conservation of Nature and Natural resources
K	potassium
KPa	kilopascals
ℓ	litre
M	molar
MeOH	methanol
Mg	magnesium
mg and mg ℓ^{-1}	milligrams and milligrams per litre
$m\ell$	millilitres
mm	millimetres
MMS	modified Murashige and Skoog medium
Mo	molybdenum
M&S or MS	Murashige and Skoog medium (1962)
NAA	napthalenacetic acid
$NaOC\ell$	sodium hypochlorite
NaOH	sodium hydroxide
N-FNGAL or NFGAL	N-formylnorgalanthamine
NH_4^+	ammonium ions
NO_2	nitrite

NO_3^-	nitrate ions
OCℓ	hypochlorite
PAC	paclobutrazol
PEDCs	pre-embryogenic determined cells
RIA	radioimmunoassay
rpm	rotations per minute
$	US dollars
sp.	species
Sporekill™	brand name for agricultural disinfectant (distributed by Hygrotech Seed)
Tween 20™	brand name for *polyoxyethylene sorbitan monolaurate* mw 1227.54
TZ	tazettine
µℓ	microlitres
µM	micromolar
UV	ultraviolet
Zn and Zn^{2+}	zinc and zinc ions
2iP and iP	*N*-(3-methyl-2-butenyl)-1*H*-purin-6-amine
2,4-D	(2,4-dichlorophenoxy) acetic acid (2,4-D, $C_8H_6O_3Cl_2$, mw 221.04)

List of tables

List of figures

List of publications

PUBLICATIONS:

FENNELL, C. W., CROUCH, N. R. and VAN STADEN, J. 2001. Micropropagation of the River Lily, *Crinum variabile* (Amaryllidaceae). *South African Journal of Botany* 67: 74-77.

FENNELL, C. W. and VAN STADEN, J. 2001. *Crinum* species in traditional and modern medicine. *Journal of Ethnopharmacology* 78: 15-26.

PAPERS PRESENTED AT LOCAL CONFERENCES:

FENNELL, C. 1994. *Crinum* spp.: plants of horticultural and medicinal importance. Paper presented at the tenth annual meeting of the NU Research Unit for Plant Growth and Development, November 1994. Department of Botany, University of Natal, Pietermaritzburg.

FENNELL, C. and VAN STADEN, J. 1995. The establishment of *Crinum* spp. *in vitro*. Paper presented at the eleventh annual meeting of the NU Research Unit for Plant Growth and Development, November 1995. Department of Botany, University of Natal, Pietermaritzburg.

FENNELL, C. and VAN STADEN, J. 1996. Crinums in culture: plantlet regeneration. Paper presented at the twelfth annual meeting of the NU Research Unit for Plant Growth and Development, November 1996. Department of Botany, University of Natal, Pietermaritzburg.

FENNELL, C. and VAN STADEN, J. 1997. Crinums in culture: *in vitro* to *ex vitrium*. Paper presented at the thirteenth annual meeting of the NU Research Unit for Plant Growth and Development, November 1997. Department of Botany, University of Natal, Pietermaritzburg.

FENNELL, C. W. and VAN STADEN, J. 1999. Crinums in culture: multiplication factors. Paper presented at the first annual meeting of the Research Centre for Plant Growth and Development, November 1999. School of Botany and Zoology, University of Natal, Pietermaritzburg.

FENNELL, C. W., ELGORASHI, E. E. and VAN STADEN, J. 2000. Alkaloid production in *Crinum* cultures. Paper presented at the second annual meeting of the Research Centre for Plant Growth and Development, November 2000. School of Botany and Zoology, University of Natal, Pietermaritzburg.

FENNELL, C. W. and VAN STADEN, J. 2001. *Crinum moorei*: Scaling up to liquid culture for mass propagation. Paper presented at the third annual meeting of the Research Centre for Plant Growth and Development, November 2001. School of Botany and Zoology, University of Natal, Pietermaritzburg.

FENNELL, C. W., ELGORASHI, E. E. and VAN STADEN, J. 2002. *Crinum moorei*: Bulbs and biotechnology. Twenty-eighth Annual Conference of the South African Association of Botanists, 13 - 16 January. Rhodes University, Grahamstown.

POSTERS PRESENTED AT LOCAL, NATIONAL AND INTERNATIONAL CONFERENCES

FENNELL, C. W. and VAN STADEN, J. 1997. The *in vitro* conservation of *Crinum moorei*. International Symposium on Biotechnology of Tropical and Subtropical species, 29 September - 3 October. Brisbane, Australia.

FENNELL, C. W. and VAN STADEN, J. 1997. The *in vitro* conservation of *Crinum moorei*. CIPUR Symposium, 30 October. University of Natal, Durban.

FENNELL, C. W. and VAN STADEN, J. 1998. Micropropagation of *Crinum moorei*. Twenty-fourth Annual Conference of the South African Association of Botanists, 12 - 16 January. Cape Town.

* (A list of paper and poster presentations that have emanated from this work.)

Chapter 1
INTRODUCTION

South Africa has a rich floral heritage that includes more bulbous plants than any other country. Some have long been cultivated in gardens all over the world while many still have the potential to be exploited commercially. The flowers are perhaps the most beautiful representatives of our South African flora and make a spectacular show when they appear after a dormant season. With this apparent disappearance and then reappearance at certain times of the year, it is not surprising that they entered into folklore. Their medicinal properties were esteemed by ancient herbalists who used them for all manner of ills. The use of plants for traditional remedies is still practised today by about two thirds of the South African population. Gatherers dig up bulbs by the thousands to sell at the *muthi* markets each week. Increasing urbanisation has transformed *muthi* into a commodity, placing enormous pressures on plants in the wild.

Crinum species are particularly vulnerable. This is because they are much sought after as ornamental plants and have a long history of ethnobotanical usage worldwide. As medicinal plants they are highly valued. As such they have attracted interest from medical science whose chemical analyses have confirmed the rationale for the plant's usage with the isolation of active compounds. The genus possesses several biological features that further exacerbate conservation efforts. That is why tissue culture may prove to be a worthwhile alternative to conventional propagation techniques for their conservation *ex situ*.

DISTRIBUTION AND MORPHOLOGY

Crinum is a large genus of 130 species which belongs to the family Amaryllidaceae. Representatives of the genus are found in the tropics of Africa, Asia and America and in the temperate regions of the northern and southern hemispheres. In the south crinums occur in South Africa, southeast Asia and Australia and in the north in Japan and the southern regions of the U.S.A. (VERDOORN, 1973; BRYAN, 1989). The centre of diversity is in Africa, south of the Sahara (FANGAN & NORDAL, 1993). More than half the number of species are known to occur in Africa. In South Africa, 21 species are found; sparsely on mountain- or hill-slopes and more commonly in low lying areas (Figure 1.1), on river banks and at the coast. Some species flourish in marshes and pans (VERDOORN, 1973).

All the species produce very large tunicated bulbs (Figure 1.2). These are rounded with a long neck; sometimes forming a false stem from the sheathing bases of old leaves. The

leaves are arranged in two rows or form a rosette. They vary in colour, width, ciliation and texture. In some species the leaf margins are ruffled. The scapes are short or long and are always produced to one side of the leaves. Each produces an umbel of 1 - 40 flowers which are amongst the largest in the Amaryllidaceae. The flowers are bell or trumpet-shaped and white, pale or deep pink in colour. They are sometimes marked with red or purple down the centre of each perianth segment, like a candy stripe (Figure 1.2). As the flowers fade they turn brown or red depending on the species. Some species, such as *C. moorei* and *Crinum neriodes,* are pleasantly scented. Others have a rather sickly smell e.g. *C. bulbispermum.* The fruit is a capsule and in some species is beaked by the persistent base of the perianth tube. Each capsule contains many irregularly-shaped or globose seeds (VERDOORN, 1973).

Figure 1.1: Crinums grow in ① grasslands, ② low lying areas near rivers and in ③ damp shade

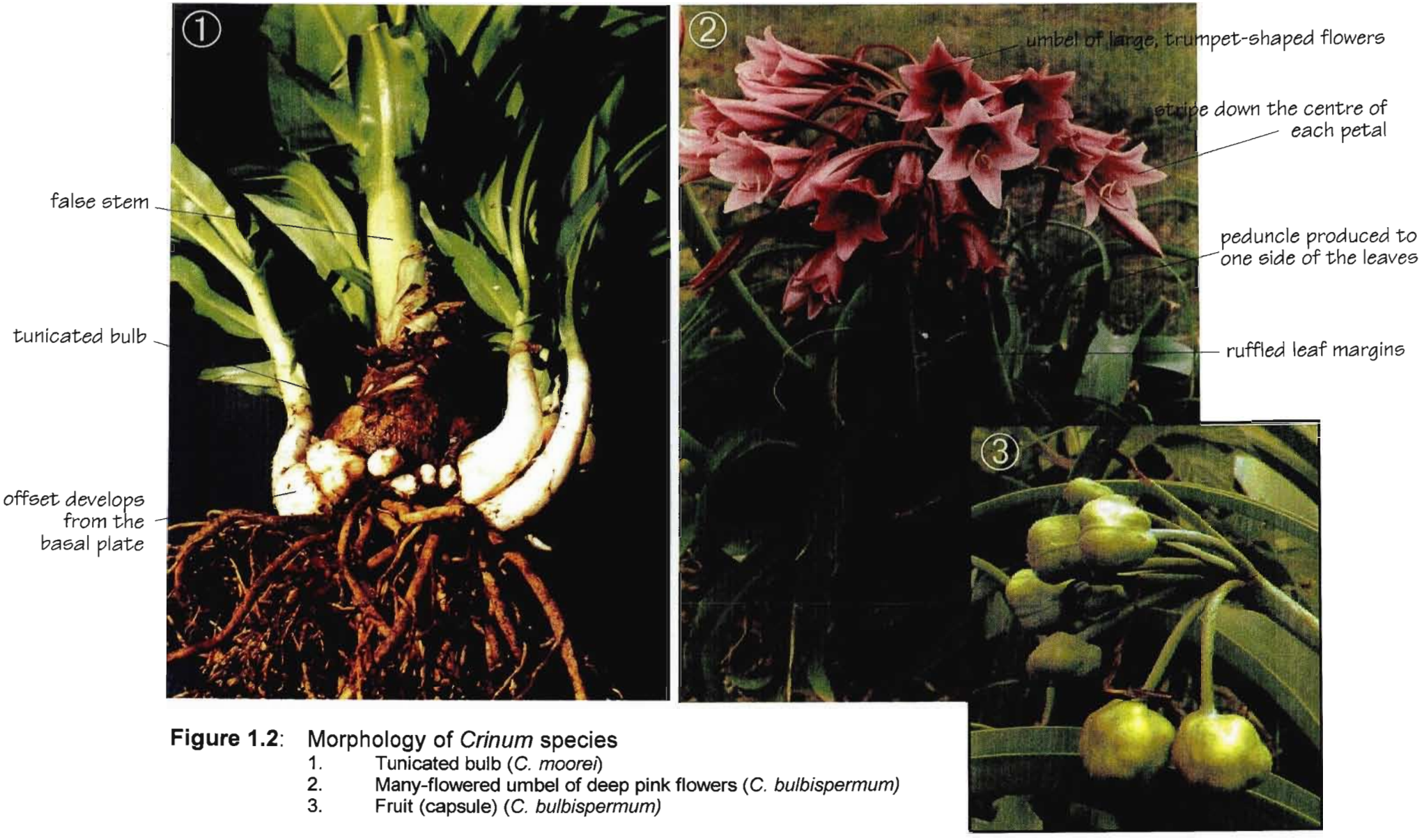


Figure 1.2: Morphology of *Crinum* species

1. Tunicated bulb (*C. moorei*)
2. Many-flowered umbel of deep pink flowers (*C. bulbispermum*)
3. Fruit (capsule) (*C. bulbispermum*)

HORTICULTURAL USE

History of cultivation

Crinums were brought into general cultivation as early as the 17th century. The first species to be introduced to English gardeners was *C. asiaticum*, from China, in 1732. This was later followed by several species native to South Africa, including *C. bulbispermum* in 1752 and *C. macowanii* and *C. moorei* in 1874 (BRYAN, 1989). Crinums were collected by travellers and explorers who found at the Cape of Good Hope a veritable treasure trove of bulbous species. The colony of Natal, too, was a paradise for the Victorian plant hunter. Early records show that shipments of plants to Kew often included crinums. The Anglican Bishop, John William Colenso, even sent crinum bulbs to Kew in exchange for Azaleas; a shrub now synonymous with Pietermaritzburg (McCRACKEN & McCRACKEN, 1990). *C. moorei* was collected by a Mr Webb while serving in the army in South Africa in the 1860's. Seed was sent to Dr. Moore of Glasnevin, Dublin (after whom the species is named) who grew the type specimen (VERDOORN, 1973).

Like many other bulbs that inspired artists and which appeared in the coat of arms and banners of the great European noble houses, the flowers of the Orange River Lily (*Crinum bulbispermum*) were depicted in stylized form in the coat of arms of the former Orange Free State.

C. moorei has long been popular as a garden plant both in South Africa and abroad (ELIOVSON, 1967) and was also known to gardeners as *C. colensoi*, *C. mackenii* and *C. natalensis* (VERDOORN, 1973) or by the common names, Natal Lily and Inanda Lily (BAYER, 1979). Apparently, it was one of the first plants cultivated by early settlers around Grahamstown (ALEXANDER, 1994).

Crinums in the garden

Sima Eliovson, in her book Wild Flowers of Southern Africa (1980), writes this of crinums: "Crinums are very desirable garden plants with their sturdy, decorative foliage and large lily-like flowers". In fact, "few plants are more striking than this stately Amaryllid" (McFARLAND *et al.*, 1948). Of all the *Crinum* species, it is the South African ones that are commonly cultivated (BRYAN, 1989). They are easy to grow, drought resistant (ELIOVSON, 1967; OLIVER, 1990) and suited to outdoor cultivation in sheltered temperate gardens (HUXLEY *et al.*, 1992). *C. bulbispermum* is a resilient variety as it is happy in different soil types and can survive long periods without water (ROBERTS, 1990; OLIVER, 1990). It is even known to survive a covering of ice in winter (ELIOVSON, 1980) and is probably the most commonly

cultivated crinum in North America (EVERETT, 1981). In a recent issue of the South African Garden and Home (May, 1994), *Crinum moorei* is reported to "make a spectacular show in the summer garden" and is "worth considering for those difficult areas under trees and on the south side of walls" (ALEXANDER, 1994). In general, crinums are "magnificent, free-flowering ornamentals ... splendid for beds and other plantings outdoors and for containers to decorate patios, terraces and similar places" (EVERETT, 1981). "In containers, in mass plantings or even in swamp gardens, the Orange River Lily makes a spectacular sight" (OLIVER, 1990). They can be effectively placed with the blue *Agapanthus* and other summer-flowering bulbs (BRYAN, 1989) and can be grown at water sides (EVERETT, 1981) for which *C. campanulatum* - an aquatic species - is often recommended.

Popular hybrids

As a group, Crinums are a tempting group to hybridize, especially the more colourful species. As far back as 1820, Dean William Herbert initiated an extensive breeding programme which yielded 24 named hybrids; only two of which produced seed. Current breeding efforts are beset with problems. For although hybrids are commonly obtained, most are usually sterile. The hybrid pollen may be viable, but unless it is parental, is unlikely to be accepted. Success, therefore, is limited due to genetic conflicts. Despite these difficulties, enthusiastic breeders have persisted in their efforts with the result that there are, today, a number of popular hybrids, both intergeneric and interspecific. *Crinodonna* is the result of crossing *Amaryllis belladonna* with *Crinum moorei* and was first described in Florence in 1921 (BRYAN, 1989). There are several colour forms including pure white. The plants are, however, rare. Chittenden (1956) also reports that hybrids between *Crinum* and *Hymenocallis* have been produced. *C.x powellii* is an interspecific hybrid between *C. bulbispermum* and *C. moorei* which is grown for its heavy umbels of sweetly fragrant flowers. There are four colour varieties (HUXLEY *et al.*, 1992). The white variety has been cultivated since 1893 (BRYAN, 1989) and has flowers of "exceptional beauty" (HUXLEY *et al*, 1992). Bryan (1989) maintains that *C.x powellii* is "one of the best for garden use" and should be the first *Crinum* to try in the garden.

Crinums as future floricultural crops

Ornamental crop production has become a highly specialised industry. This has resulted in an increased demand for new species and cultivars. As "bulbs are one of nature's most intriguing creations - ready-made flowers squeezed into a convenient package" (BLACKER, 1994) they are of great importance to the horticultural trade. South Africa has 2700 species of bulbs; more than any other single country (BLACKER, 1994). Some have been long cultivated in countries all over the world e.g. *Freesia* and *Gladiolus*. However, it appears that

new cultivars were developed from a limited number of species (STIRTON, 1980). Thus, South Africa still "remains an untapped reservoir of horticultural importance" (STIRTON, 1980) with "much scope for the selection and development of cultivars both from the wild state and from garden collections" (STIRTON, 1980). Locally, Cunningham believes there is a need to develop crops that are suited to areas of low rainfall which would benefit small-scale producers in the rural areas (CUNNINGHAM *et al.*, 1993).

Jansen Van Vuuren *et al.* (1993), in a paper entitled "South African flowering plants with a potential as future floriculture crops" cites *Crinum* species as geophytes with "great potential to be developed as cut flowers and/or pot plants". This is because they are hardy and produce large numbers of attractive blooms (SLABBERT, 1989) for many months of the year (BRYAN, 1989). Bryan (1989) lists *Crinum* species as "especially good cut flowers, worthy of being grown for that purpose". They "make excellent long-lasting cut flowers" (MATHEW & SWINDELLS, 1995). Some are even scented (SLABBERT, 1989); a quality that is elusive amongst flowers (BRYAN, 1989). *C. moorei*, for example, is known to be very fragrant as are the flowers of *C. acaule* which are delicately perfumed like carnations (NICHOLS, 1984). Those of *C. minimum* are sweetly scented like frangipani flowers (CRAIB, 1997).

Many of the smaller South African crinums readily cross and produce semi-dwarf hybrids that do not multiply vegetatively. Both wild types and hybrids yield "a promising selection of bulbs ideally suited for pot and tub cultivation" (LEHMILLER, 1996). Another factor in their favour is the fact that most African crinums are indigenous to regions outside of the tropical rain forests (LEHMILLER, 1996). Wild species like these are especially sought after as collector's items (KOOPOWITZ, 1986). Duncan (1982) maintains that *Crinum neriodes* would make an interesting pot-subject for collectors of unusual wild flowers, if it were readily available. *Crinum acaule* is another. For this "exciting plant(s) from northern Zululand and Tongoland" (NICHOLS, 1984) "is undoubtedly one of the most spectacular species of *Crinum*" (ARCHER, 1997).

ETHNOBOTANY

For centuries, ethnic tribes throughout the world have used bulbs extensively in witchcraft and herbal remedies (ELIOVSON, 1967) - a tradition that continues to this day. In South Africa, traditional medicine practices are as old as the Khoikhoi and Bushmen tribes. These and several other indigenous peoples in South Africa, have for some time used plants of the Amaryllidaceae in medicinal preparations (WATT & BREYER-BRANDWIJK, 1962; HUTCHINGS *et al.*, 1996). Although the knowledge survived for centuries through apprenticeship to traditional healers, early missionaries, colonial botanists and Victorian plant hunters were among the first to document it (BRYANT, 1966; WATT & BREYER-BRANDWIJK, 1962).

Reports in the literature suggest widespread use of *Crinum* species in treating a variety of ailments (FENNELL & VAN STADEN, 2001) (Table 1.1). In some cases the plants were and are used in different countries for the same medicinal purposes. In South Africa, *Crinum bulbispermum* is a favourite medicinal herb (ROBERTS, 1990). The southern Sotho use the leaves and sliced or crushed bulbs to make a strong brew for treating colds, coughs, and as an external application or wash for wounds, scrofula and haemorrhoids. A drawing poultice for abscesses and suppurating sores is also made from the bulbs. These may be roasted by the Zulu and Tswana and applied to aching joints. Rheumatism and backache are treated in a similar way. The Zulus bind the roasted bulb to varicosities using the leaves which, because of their strap-like shape, are also used to bind dressings in place. The flowers are bound over swollen joints and sprains to soothe and help reduce swelling. Several tribes are reported to use the juice squeezed from the base of the leaves to cure earache (WATT & BREYER-BRANDWIJK, 1962; ROBERTS, 1990). Sometimes pieces of roasted bulb are placed behind the ear or over the ear to ease the pain. Roberts (1990) reports that some tribes make a brew of the leaves which they believe to be an effective treatment for malaria. This same brew is drunk by the Zulu as a treatment for rheumatic fever (usually ½ cup of chopped leaves in 1 cup boiling water, strained after standing for 5 minutes). The Tswana drink a brew of crushed leaf bases and stalks to increase the flow of urine in bladder and kidney infections. The sliced bulb is also warmed and applied over the kidneys to ease discomfort (ROBERTS, 1990). Hutchings (1989) records its use as a Zulu, Xhosa and Sotho gynaecological remedy and charm. An infusion of unspecified plant parts is taken during pregnancy to ensure an easy delivery (HUTCHINGS *et al.*, 1996). In countries outside South Africa, *C. bulbispermum* is known to increase milk flow (JACOT GUILLARMOD, 1971) and as a treatment for rheumatism, haemorrhoids and sores and abscesses (WATT & BREYER-BRANDWIJK, 1962).

Crinum delagoense and *C. moorei* bulbs are used in Zulu and Xhosa traditional medicine, to prepare a decoction for treating swellings and urinary tract problems (HUTCHINGS *et al.*, 1996). Preparations of *C. macowanii* also appear to be used by the Zulu, Xhosa and Sotho for swellings, urinary tract problems (HUTCHINGS *et al.*, 1996) and skin complaints. The Xhosa use the fluid from the bulb to soothe itchy skin rashes (HUTCHINGS & JOHNSON, 1986). Pujol (1990) cites a number of principal uses for *C. macowanii*, namely: pus diseases, blood cleansing, kidney and bladder diseases, glandular swelling, fever, infected sores, boils and acne. The plant fibres are used in southern Africa as poultices and bandages (HUTCHINGS *et al.*, 1996). In Zimbabwe, this species is prescribed by traditional medical practitioners for backache, where it is applied as a compress, as an emetic, for increasing lactation in both animals and humans (MAVI, 1994), venereal diseases and increasing blood supply (GELFAND *et al.*, 1985; DURI *et al.*, 1994).

Six species of the Natal Lily or *Crinum* are used for colds, leprosy and scrofula (GITHENS, 1949; WATT & BREYER-BRANDWIJK, 1962; BRYANT, 1966). Bryant, in a book entitled "Zulu medicine and medicine men" (1966), describes the treatment for scrofula which included a mixture of *umDuze* (crinum) (BRYANT, 1905) and other roots. These were chopped, pounded and boiled in a small quantity of water to form a decoction of blood-purifying drugs, called an *imBhiza*. A dessert-spoonful was taken every morning and evening. Githens (1949) also includes several *Crinum* species in a list of plants containing alkaloids used as tonics, febrifuges, antiperiodics and astringents. The Zulu people use crinum bulbs in medicines for the treatment of rheumatic fever and difficulty in micturition (WATT & BREYER-BRANDWIJK, 1962). To allay the pains of rheumatic fever, a decoction of *Gunnera perpensa* roots and Natal Lily bulbs is taken (BRYANT, 1966). Difficulty in urination is treated with a mixture of *Gunnera perpense* and *Crinum* roots. These are either chopped or pounded and boiled in water to make a decoction (BRYANT, 1966). Before pregnancy, Zulu women, prepare an *isihlambezo* of *Crinum* bulbs, *Gunnera perpensa* and *Rhoicissus tridentata* (isihlambezo). Cunningham and Zondi (1991) report that a similar preparation is used to treat the retained placenta in cows. According to Zulu folklore, *Crinum* species are used in combination with *Eucomis, Boweia, Xanthozylum* and *Becium* as a form of chemotherapy for cancer (ALBRECHT *et al.*, 1996).

The use of crinums extends to animal treatment. *Crinum* species, including *C. delagoense*, *C. macowanii* and *C. moorei* are used by the Zulus to treat weight loss, low milk production, milk loss, healthy calves and in the treatment of retained placenta among cattle (HUTCHINGS, 1989; CUNNINGHAM & ZONDI, 1991).

The leaves of *Crinum* sp. are reported to be browsed by cattle (WATT & BREYER-BRANDWIJK, 1962) e.g. *Crinum bulbispermum* (ROBERTS, 1990) and are also known to provide a tasty meal for herbivores, birds and mammals that forage amongst the vegetation of the wetlands (BRISTOW, 1988). In an article by J. Friedberg in the Landbouweekblad, 4th May 1971, *Crinum paludosum* is mentioned as a valuable cattle feed, particularly for the dry S.W.African country, as it can be browsed for several months after flowering (VERDOORN, 1973).

It is estimated that 64% of the total world population depends on traditional medicine for their primary health care (FARNSWORTH, 1994 and SINDIGA, 1994; cited by COTTON, 1996). In Africa, where there is a severe shortage of qualified personnel in modern medicine (MAMMEM & CLOETE, 1996) and imported pharmaceuticals are expensive (SCOTT, 1993), this figure is even higher. Here, 80% of the people rely on plant remedies prescribed by traditional healers (HAMILTON, 1993). Most (75%) black South Africans regularly consult traditional medical practitioners (STREAK, 1995). The Zulus believe that the plant-based

medicines have power (amandla) and so traditional medical practices must also be seen in an historical and cultural perspective (CUNNINGHAM, 1984).

Table 1.1: The ethnobotany of *Crinum* species

Crinum spp.	Use	Country	Reference
C. amabile	Emetic Rheumatism Earache	Vietnam	Pham *et al.*, 1998
C. asiaticum	Anodyne	Malaya	Beckstrom-Sternberg *et al.*, 1994
	Antidote	Somoa	Beckstrom-Sternberg *et al.*, 1994
	Antidote (arrow poison)	Java	Beckstrom-Sternberg *et al.*, 1994
	Bilious		Beckstrom-Sternberg *et al.*, 1994
	Carbuncle	China	Beckstrom-Sternberg *et al.* 1994
	Chafing	Malaya	Beckstrom-Sternberg *et al.*, 1994
	Diaphoretic	Egypt, Southeast Asia and Polynesia	Beckstrom-Sternberg *et al.*, 1994 Etkin, 1986
	Dysuria	Java	Beckstrom-Sternberg *et al.*, 1994
	Edema	Java	Beckstrom-Sternberg *et al.*, 1994
	Emetic	Egypt, Somoa and Malaya Southeast Asia and Polynesia	Beckstrom-Sternberg *et al.*, 1994 Etkin, 1986
	Expectorant		Beckstrom-Sternberg *et al.*, 1994
	Fever	Malaya	Beckstrom-Sternberg *et al.*, 1994 Ahmad, 1996
	Headache	Malaya	Beckstrom-Sternberg *et al.*, 1994 Ahmad, 1996
	Ipecac	India	Beckstrom-Sternberg *et al.*, 1994
	Laxative		Beckstrom-Sternberg *et al.*, 1994
	Lumbago	Malaya	Beckstrom-Sternberg *et al.*, 1994 Ahmad, 1996
	Orchitis	Malaya	Beckstrom-Sternberg *et al.*, 1994
	Sores	Malaya	Beckstrom-Sternberg *et al.*, 1994
	Swelling	Malaya	Beckstrom-Sternberg *et al.*, 1994 Ahmad, 1996
	Tonic		Beckstrom-Sternberg *et al.*, 1994

	Tumour (stomach)	Zaire, Indochina	Beckstrom-Sternberg *et al.*, 1994
	Whitlow	India	Beckstrom-Sternberg *et al.*, 1994
	Wound	Philippines	Beckstrom-Sternberg *et al.*, 1994
	Yaws (preventative)	Java	Beckstrom-Sternberg *et al.*, 1994
C. bracteatum	Whitlow	India	Beckstrom-Sternberg *et al.*, 1994
C. bulbispermum	Abscesses and sores	South Africa	Watt & Breyer-Brandwijk, 1962
	Aching joints	South Africa (Zulu & Tswana)	Roberts, 1990
	Backache	South Africa	Watt & Breyer-brandwijk, 1962
	Binding for dressings	South Africa	Watt & Breyer-Brandwijk, 1962
	Cattle feed	South Africa	Roberts, 1990
	Charm	South Africa (Zulu, Xhosa, Sotho), Lesotho	Hutchings, 1989 Jacot Guillarmod, 1971; Beckstrom-Sternberg *et al.*, 1994
	Colds	Lesotho Southern Sotho South Africa	Beckstrom-Sternberg *et al.*, 1994; Roberts, 1990 Githens, 1949
	Coughs	Southern Sotho	Roberts, 1990
	Earache	South Africa	Watt & Breyer-Brandwijk, 1962
	Gynaecological remedy	South Africa (Zulu, Xhosa, Sotho)	Hutchings, 1989
	Haemorrhoids	Southern Sotho	Watt & Breyer-Brandwijk, 1962; Roberts, 1990
	Kidney and bladder infections (increase urine flow)	South Africa (Tswana)	Watt & Breyer-Brandwijk, 1962
	Lactogogue	Lesotho	Beckstrom-Sternberg *et al.*, 1994
	Malaria	South Africa	Watt & Breyer-Brandwijk, 1962
	Reduce swelling of swollen joints and sprains	South Africa	Watt & Breyer-Brandwijk, 1962
	Scrofula	Southern Sotho South Africa	Roberts, 1990 Githens, 1949
	Varicosities	South Africa (Zulu)	Watt & Breyer-Brandwijk, 1962
	Wounds	Southern Sotho	Roberts, 1990

C. cochinchinense	Diaphoretic	Madagascar	Githens, 1949
	Emetic	Madagascar	Githens, 1949
C. defixim	Burns		Beckstrom-Sternberg *et al.*, 1994
	Carbuncle		Beckstrom-Sternberg *et al.*, 1994
	Poison		Beckstrom-Sternberg *et al.*, 1994
	Whitlow		Beckstrom-Sternberg *et al.*, 1994
C. deflexum	Whitlow	India	Beckstrom-Sternberg *et al.*, 1994
C. delagoense	Urinary tract infections	South Africa (Zulu and Xhosa)	Hutchings *et al.*, 1996
	Swellings of the body	South Africa (Zulu)	Hutchings & Johnson, 1986
	Veterinary treatment of retained placenta, milk loss, low milk production, healthy calves and weight loss in cattle	South Africa (Zulu)	Hutchings, 1989 Cunningham & Zondi, 1991
C. erubescens	Asthma	Haiti	Beckstrom-Sternberg *et al.*, 1994
	Bronchitis	Haiti	Beckstrom-Sternberg *et al.*, 1994
	Cardiotonic	Haiti	Beckstrom-Sternberg *et al.*, 1994
	Emetic	Dominican Republic	Beckstrom-Sternberg *et al.*, 1994
	Expectorant	Haiti	Beckstrom-Sternberg *et al.*, 1994
	Malaria	Haiti	Beckstrom-Sternberg *et al.*, 1994
	Resolvent	Haiti	Beckstrom-Sternberg *et al.*, 1994
C. firmifolium	Parasitic skin diseases	Madagascar	Razafimbelo *et al.*, 1996
C. flaccidum	Starch for gruel	Australia	Usher, 1974
C. foetidum		Botswana (Kalahari or !ko Bushmen)	Heinz & Maguire
C. giganteum	Ceremonies and festivals	Mexico (Sierra Norte)	Leszczynska-Borys, 1995
	Leprosy	Congo	Githens, 1949 Watt & Breyer-Brandwijk, 1962
C. jagus	Anticonvulsant	Nigeria	Adesanya *et al.*, 1992
	Open sores	Nigeria	Adesanya *et al.*, 1992
	Veterinary	Cameroon	Bizimana, 1994

C. kirkii	Indigestion	East Africa	Githens, 1949
	Purgative	Tanganyika / Tanzania	Watt & Breyer-Brandwijk, 1962
	Rat poison	Tanganyika / Tanzania	Watt & Breyer-Brandwijk, 1962
	Sores (wash)	Kenya (Bondei)	Watt & Breyer-Brandwijk, 1962 Bastida *et al.*, 1995
	Making shamba boundaries	Kenya	Bastida *et al.*, 1995
C. latifolium	Earache		Usher, 1974 Beckstrom-Sternberg *et al.*, 1994
C. latifolium	Fistula	India (Santal)	Beckstrom-Sternberg *et al.*, 1994
	Rheumatism	India	Usher, 1974 Beckstrom-Sternberg *et al.*, 1994
	Rubefacient	India	Beckstrom-Sternberg *et al.*, 1994
	Tubercle	India (Santal)	Beckstrom-Sternberg *et al.*, 1994
	Tumour	Indochina	Beckstrom-Sternberg *et al.*, 1994
	Whitlow	India	Beckstrom-Sternberg *et al.*, 1994
C. longiflorum	Poison	USA	Beckstrom-Sternberg *et al.*, 1994
C. macowanii	Acne	South Africa	Pujol, 1990
	Veterinary treatment of retained placenta, milk loss, low milk production, healthy calves and weight loss in cattle veterinary	South Arica (Zulu) Zimbabwe	Cunningham & Zondi, 1991 Bizimana, 1994
	Backache	Zimbabwe	Gelfand *et al.*, 1985 Duri *et al.*, 1994
	Bandages and poultices	Southern Africa	Hutchings *et al.*, 1996
	Blood cleansing and increased supply	South Africa Zimbabwe	Pujol, 1990 Gelfand *et al.*, 1985
	Boils	South Africa	Pujol, 1990
	Emetic	Zimbabwe	Gelfand *et al.*, 1985 Duri *et al.*, 1994
	Fever	South Africa	Pujol, 1990
	Glandular swelling	South Africa	Pujol, 1990

	Kidney and bladder diseases	South Africa	Pujol, 1990
	Lactation (humans and animals)	Zimbabwe	Gelfand *et al.*, 1985 Duri *et al.*, 1994 Mavi, 1994
	Pus diseases	South Africa	Pujol, 1990
	Skin complaints e.g. rashes	South Africa (Zulu, Xhosa, Sotho)	Pujol, 1990 Hutchings *et al.*, 1996
	Sores	South Africa	Pujol, 1990
	Swellings and urinary tract problems	South Africa (Zulu)	Hutchings *et al.*, 1996
	Venereal diseases	Zimbabwe	Gelfand *et al.*, 1985
C. macrantherum	Emetic	New Guinea	Beckstrom-Sternberg *et al.*, 1994
	Wounds	New Guinea	Beckstrom-Sternberg *et al.*, 1994
C. moorei	Veterinary treatment of retained placenta, milk loss, low milk production, healthy calves and weight loss in cattle veterinary	South Africa (Zulu)	Hutchings, 1989 Cunningham & Zondi, 1991
C. paludosum	Cattle feed	South Africa and Namibia	Verdoorn, 1973
C. pratense	Intestinal diseases (diarrhoea and dysentry)	India	Prance *et al.*, 1994
C. scabrum	Leprosy	Congo	Githens, 1949
Crinum species	Antidote to magical poisons	New Guinea Highlands	Etkin, 1986
	Antiperiodic		Githens, 1949
	Astringent		Githens, 1949
	Cancer	South Africa (Zulu)	Albrecht *et al.*, 1996
	Colds		Githens, 1949
	Dyspepsia	New Guinea	Beckstrom-Sternberg *et al.*, 1994
	Emetic		Le Maout & Decaisne, 1873
	Febrifuge		Githens, 1949
	Leprosy		Githens, 1949

	Micturition	South Africa (Zulu)	Watt & Breyer-Brandwijk, 1962 Bryant, 1966
	Pregnancy (before)	South Africa (Zulu)	Cunningham & Zondi, 1991
	Rheumatic fever	South Africa	Watt & Breyer-Brandwijk, 1962 Bryant, 1966
	Scrofula	South Africa (Zulu)	Githens, 1949 Watt & Breyer-Brandwijk, 1962 Bryant, 1966
	Sores	New Guinea Highlands	Etkin, 1986
	Stomachache	New Guinea	Beckstrom-Sternberg *et al.*, 1994
	Tanning leather	Southern Africa (Bushmen of the Kalahari)	Van der Post & Taylor, 1984
	Tonic		Githens, 1949
	Tumours	Asia and America	Le Maout & Decaisne, 1873
	Vomiting (induction)	New Guinea Highlands	Etkin, 1986
C. yuccaeflorum	Rubefacient (topically irritating)	West Africa	Githens, 1949
C. zeylanicum	Cataplasm	Dominican Republic	Beckstrom-Sternberg *et al.*, 1994
	Malaria	Dominican Republic	Beckstrom-Sternberg *et al.*, 1994
	Pectoral	Dominican Republic	Beckstrom-Sternberg *et al.*, 1994
	Poison	Moluccas	Le Maout & Decaisne, 1873

PHARMACEUTICAL USE

Although once regarded as of little relevance to contemporary drug discovery, traditional remedies are now proving to be an important source of potentially therapeutic drugs (COX & BALICK, 1994). In fact, three quarters of the 120 plant-based drugs in common use were discovered by following leads provided by traditional medicine in various parts of the world (SCOTT, 1993). Today, the ethnobotanical approach to drug discovery is perhaps the most productive strategy in screening some 265 000 flowering plant species as indigenous usage provides powerful clues to a plant's biological activity (COX & BALICK, 1994). A single South African pharmaceutical company screened 600 traditional medicines and found that 80% were pharmacologically active (STREAK, 1995). Fourie *et al.* (1992; cited by CUNNINGHAM *et al.*, 1993) also maintain that the majority of traditional medicine plants possess some kind of pharmacological activity. Since South Africa has a wealth of traditional herbal remedies, the indigenous flora may, in the future, offer many medical breakthroughs (STREAK, 1995).

Amaryllidaceae alkaloids

The ethnobotanical use of *Crinum*, as with many other medicinal plants, can be explained on the basis of chemical and physiological studies. In most cases these confirm the therapeutic value of the plants. However, where plants have not been studied chemically, a plant's potential pharmaceutical value can be assumed if it is used widely i.e. in different geographical regions for the same purpose. When different species are used for the same healing properties, it may be that they contain a common ingredient (GITHENS, 1949).

The reason why Crinums are used for medicinal purposes and in a number of countries for similar reasons, is possibly due to their alkaloidal constituents, which in some instances are common to a variety of species. Phytochemically, the genus has already yielded more than 170 different compounds; mostly alkaloidal in nature. Classically these are grouped as the Amaryllidaceae alkaloids because of their limited taxonomic distribution. Only Amaryllids have alkaloids of the norbelladine type. Derivatives of norbelladine (an intermediate en route to the majority of Amaryllidaceae alkaloids) can, in fact, be used as taxonomic characters for the determination of plant relationships among the Amaryllidaceae (WALLER & NOWACKI, 1978). *Crinum* species contain the phenanthridine alkaloids like *Galanthus* and *Narcissus* - other members of the Amaryllidaceae (SPOERKE & SMOLINSKE, 1990). Amine, carboline, isoquinoline, lactone and other uncharacterised alkaloids are also reported to occur in *Crinum* species (GLASBY, 1991). A list of the trivial names of *Crinum* alkaloids, their distribution and biological activities are reviewed by Ghosal *et al.* (1985). A variety of new alkaloids have since been reported, while others have been isolated for the first time from other species (BASTIDA *et al.*, 1995; BENTLEY, 1986; BEUTNER & FRAHM, 1986; ELGORASHI & VAN STADEN, 2001; ELGORASHI *et al.*, 1999, 2001[a] and 2001[b]; GRUNDON, 1984, 1985, 1987, 1989; HOSHINO, 1998; LEWIS, 1990, 1992, 1993, 1994, 1996, 1997, 1998, 1999, 2000; LIKHITWITAYAWUID *et al.*, 1993; MACHOCHO *et al.*, 1998; NAIR *et al.*, 1998 and 2000; PHAM *et al.*, 1998; RAMADAN *et al.*, 2000; RAZAFIMBELO *et al.*, 1996; VELTEN *et al.*, 1998; VILADOMAT *et al.*, 1997).

Biological activity

The alkaloids are structurally diverse and possess an array of physiological activity "unrivalled by any other group of natural products" (HARBORNE & BAXTER, 1993). In the plant they play a role in the protective and repair mechanisms and assist in the biosynthesis of compounds needed for cell to cell communication (GHOSAL *et al.*, 1990).Their medicinal properties, in particular, have attracted much interest.

Within the Amaryllidaceae group, the *Crinum* alkaloids exhibit a range of biological activity (FENNELL & VAN STADEN, 2001), both pharmacological and microbiological (PHAM *et al.*, 1998) (Table 1.2). Among the most noted effects are: analgesic, central nervous system, antitumour, antiviral (LEWIS, 1990) and anticholinergic (PHAM *et al.*, 1998). This has stimulated further pharmacological screening of the alkaloids (LEWIS, 1994) which more recently showed activity against HIV (PHAM *et al.*, 1998).

Table 1.2: Biological activity of the Amaryllidaceae and *Crinum* alkaloids

Biological activity	References
Analgesic and Nervous system effects	Bonner, 1995 Cordell, 1981 Eichhorn *et al.*, 1998 Ghosal *et al.*, 1985 Githens, 1949 Greenblatt *et al.*, 1999 Harborne & Baxter, 1993 Lewis, 1996, 1998 and 1999 Martin, 1987 Sramek *et al.*, 2000 Wildman, 1960
Anticancer activity	Abd-el-hafiz *et al.*, 1991 Ahmad, 1996 Antoun *et al.*, 1993 Fuganti, 1975 Ghosal *et al.*, 1985 Grundon, 1984 Harborne & Baxer, 1993 Lewis, 1990 Likhitwitayawuid *et al.*, 1993 Manske, 1975 Martin, 1987 Nair *et al.*, 1998 Spoerke & Smolinske, 1990 Viladomat *et al.*, 1995 Wagner *et al.*, 1988 Weniger *et al.*, 1995 Wildman, 1960
Immunostimulatory activity	Ghosal *et al.*, 1985
Antifertility activity	Wildman, 1960 Chattopadhyay *et al.*, 1983
Anti-infective activity *Antibacterial and antifungal activity*	Adesanya *et al.*, 1992 Chaumont *et al.*, 1978 Cordell, 1981 Ghosal *et al.*, 1985 Gundidza, 1986
Antimalarial activity	Likhitwitayawuid *et al.*, 1993 Viladomat *et al.*, 1995 Watt & Breyer-Brandwijk, 1962
Antiparasitic activity	Machocho *et al.*, 1998

Antiviral activity	Duri *et al.*, 1994 Ghosal *et al.*, 1985 Harborne & Baxer, 1993 Ieven *et al.*, 1983 Lewis, 1990 Martin, 1987 Vrijsen *et al.*, 1986
Cardiovascular activity	Harborne & Baxer, 1993 Martin, 1987 Wildman, 1960
Respiratory system effects	Harborne & Baxer, 1993 Martin, 1987
Emetic and diaphoretic activity	Etkin, 1986 Spoerke & Smolinske, 1990
Insecticidal effects	Harborne & Baxter, 1993 Martin, 1987 Singh & Pant, 1980 Singh *et al.*, 1996
Antiviral activity in plants	Frahmy & Mohamed, 1992
Antifungal activity in plants	Cook & Loudon, 1954 Etkin, 1986 Waller & Nowacki, 1978
Growth regulation in plants	Harborne & Baxter, 1993 Martin, 1987 Wildman, 1960

Other compounds and their biological effects

A number of other products have been isolated from *Crinum* bulbs and seeds. These include: neokestose (an oligosaccharide) (YASUDA *et al.*, 1986), phenolics (EL-HAFIZ *et al.*, 1990; RAMADAN *et al.*, 2000), flavonoids (ALI *et al.*, 1981; ALI *et al.*, 1988 [flavans]; ABD-EL-HAFIZ, 1990), mucilage (ABD-EL-HAFIZ, 1990), aliphatic hydroxyketones, methyl palmitate, palmitic acid and stearic acid (ABD-EL-HAFIZ, 1991), keto alcohols (ABD-EL-HAFIZ, 1990), esters, steroids and triterpenoids (GLASBY, 1991), organic acids, an haemolytic sapogenin (saponin) and vanillin and coumarin (WATT & BREYER-BRANDWIJK, 1962). Polysaccharides were found in the bulbs of *C. amabile* and may be connected with the medicinal uses of the bulbs (MURAV'-EVA & POPOVA, 1989), while other extracts were shown to have antimutagenic properties (KALAYCIOGLU & ONER, 1994).

Unidentified extracts of *Crinum* species have been found to be slightly effective against experimental malaria (WATT & BREYER-BRANDWIJK, 1962) while those of *C. glaucum* caused relaxation of the gastrointestinal smooth muscle of guinea pigs (OKPO & ADEYENI, 1998).

Interest has been shown in plant lectins from members of the Amaryllidaceae. Balzarini *et al.* (1991) found that two plant lectins inhibited infection of MT-4 cells by the human immunodeficiency viruses HIV-1 and HIV-2.

Future phytochemical research

Only about 15% of the known plant species have been screened for therapeutic potential. And of these, 1% have been examined exhaustively (SCOTT, 1993). Members of the Amaryllidaceae continue to yield novel compounds "having interesting biological activity" (MARTIN, 1987). Yet plants still remain the "sleeping giant of drug development" (SCOTT, 1993) as pharmaceutical companies are slow to take up the challenge. Thus "isolation of additional alkaloids and further testing may well produce fertile fields for pharmacological research" (WILDMAN, 1960). Both local and global populations would benefit in terms of improved health care and economics (COTTON, 1996).

Worldwide, only 27 *Crinum* species (an estimated 20% of the total number of species in the genus) are represented in phytochemical analyses. Of the 21 South African *Crinum* species, only eight (*C. bulbispermum, C. buphanoides, C. delagoense, C. kirkii, C. lugardiae, Crinum macowanii, C. moorei* and *C. variabile*) have been investigated for alkaloids. The South African species, in particular, remain an untapped reservoir of potentially important biologically active compounds. This is because the centre of diversity lies south of the Sahara; a region that has a long history of traditional plant usage. Hybrids are known to contain compounds not present in either parents (JOHN,1961). Thus the possibility exists that new hybrids may yield novel compounds. The *Crinum* alkaloids would be of considerable interest to chemists studying the Amaryllidaceae alkaloids, since they are unique to the genus. "At the genus level, it appears that specific alkaloids often are associated with the *Crinum, Haemanthus, Hymenocallis* and *Narcissus* genera, but no such uniformity is noted in the species of the *Nerine* genus" (WILDMAN, 1960). Thus, phytochemical profiles of individual species may help resolve some of the taxonomic confusion (FANGAN & NORDAL, 1993), which exists because the genus has not been revised over its entire distribution range (SNIJMAN & LINDER, 1996).

Although crinums are known to contain alkaloids which, in some cases have been pharmacologically tested, "nothing is known of the effect that *Crinum* based preparations may have on livestock" (CUNNINGHAM & ZONDI, 1991).

CONSERVATION STATUS

Plant extinction: a global crisis

Wild plants are disappearing at an alarming rate. The World Conservation Monitoring Centre estimates that 10% of the world's flora are currently under threat. There is general consensus that in the next 50 years, 10% - 25% of the higher plants will be in danger of becoming extinct (KOOPOWITZ, 1986). Industrial development, air and water pollution, farming and livestock grazing and the clearance of land for timber and crop production as well as for firewood put great pressures on indigenous floras (MARSHALL, 1993; cited by NEWTON & BODASING, 1994).

Bulbs in danger

Trade in bulbs is centuries old (READ, 1989). Today, US$470 million is spent on flower bulbs in America alone (NEWTON & BODASING, 1994). And still the horticultural world looks for novelty - the source of which is nature (READ, 1989). Evidently there is growing concern for the preservation of bulbs in countries where they are being collected from the wild to meet the demands by the horticultural world for novelty plants. Many beautiful bulbs are rare or even threatened with extinction. This is especially so of the petaloid monocotyledons which are vulnerable to over collection and exploitation because they are "showy plants" (STIRTON, 1980). Because they are so easily dug up, "they have suffered more than any other (group of) plants from being reduced in numbers" (ELIOVSON, 1967).

Amaryllidaceae and the genus *Crinum*: threatened species

The Amaryllidaceae are included in a list of 17 plant families with a high proportion of threatened and rare species (HALL *et al.*, 1980). South Africa has 22% of the world's amaryllids of which 66 species (about 13 genera) have been placed in various categories of endangerment. This represents 30% of the South African species, some of which are endemic (HALL *et al.*, 1980). In areas with even higher levels of endangerment, this estimate may be conservative (KOOPOWITZ, 1986). In Natal, the family is listed as specially protected under the Natal Provincial Ordinance 14 of 1974 (CUNNINGHAM, 1988) and the KwaZulu-Natal Nature Conservation Bill as amended in 1999 (SCOTT-SHAW, 1999). It is against the law to collect bulbs or seeds of *Crinum* without a permit (LEHMILLER, 1987). Ten *Crinum* species are threatened in South Africa (HALL *et al.*, 1980). Both *C. campanulatum* and *Crinum lineare* are listed as rare (HILTON-TAYLOR, 1996). *C. campanulatum* is restricted to a few shallow pans in Alexandria, Bathurst and Peddie. "When they come into bloom en masse they are truly one of the great sights of the flower world" (BATTEN, 2000; cited by WILLIAMS, 2000). "They emerge from a dustbowl. There is nothing there and then the rains

come, and there is this vista of beauty. They must be preserved at all costs" (BATTEN, 2000; cited by WILLIAMS, 2000). *C. bulbispermum* is endangered and possibly extinct in Lesotho. This same species together with *C. graminicolum* and *C. macowanii* are reportedly rare in Swaziland. *C. mauritanium* is endangered in Madagascar (HEDBERG, 1979) and appears on the endangered list of the IUCN (EMANOIL, 1994).

Contributing factors to the species loss

Habitat destruction

The major cause of this species loss is the human population explosion and the attending destruction of habitats (KOOPOWITZ, 1986). Sandton, where once *C. graminicola* grew abundantly, is just another high rise city (OLIVIER, 1983). In Zululand, the natural habitat of *C. acaule* is densely settled and afforested with exotics. Although it survives in these plantations, it does not flower and produce seed (CRAIB & BLACKMORE, 1997). Local endemics are particularly sensitive to damage. The problem is even more acute when plants need a specialised habitat, such as a wetland. Since these are in themselves under threat, the future of plants like *C. acuale* is only "assured as long as the Greater St. Lucia Wetland Park remains" (CRAIB & BLACKMORE, 1997).

The trade in medicinal plants

In South Africa, the greatest demand for medicinal plants is in Natal. Here, the dense population is placing increasing pressures on the environment. With increasing urbanisation, there is a concomitant increase in the market for medicinal plants. "Urbanisation has transformed *muthi* into a commodity" (STREAK, 1995). Nearly 200 000 bulbs are traded at Durban's medicine markets each week (STREAK, 1995). Traders in the Witwatersrand buy wholesale from Natal Herbs and Muti Medicines while some travel on a regular basis to Durban to buy from the markets there (WILLIAMS, 1996). Crinum bulbs are one of the more commonly sold species of bulbs, roots and tubers (CUNNINGHAM, 1988; CUNNINGHAM, 1990). Known locally as *umDuze*, they are packaged in 50 kg maize bags and sold by gatherers at the main urban market in Durban for R10 (CUNNINGHAM, 1988). The market for *muthi* plants such as these is estimated to be more than R120 million a year in KwaZulu Natal alone. This figure is based on the conservative assumption that 30% of the population spends R10 a year on traditional medicines (SHERRIFFS, 1995). Collecting plants for trade is a problem as it places a strain on certain species in the wild (HAMILTON, 1993). This is because plants are harvested in an unsustainable way. Whole plants are destroyed when they are collected for their roots, bulbs or tubers. Added to this is the fact that gatherers are unskilled and take as much as possible (SHERRIFFS, 1995). Geophytes, such as *Crinum*,

are generally regarded as scarce by the herbal traders because of the manner in which they are harvested. In "An investigation of the herbal medicine trade in Natal/Kwazulu", Cunningham (1988) lists *C. moorei, C. macowanii* and *C. delagoense* as "species whose status is uncertain, but which appear to be heavily exploited and for which more data is required". Roberts (1990) writes that *C. bulbispermum* "is so prized and so used that I always fear it will become even rarer". Despite legislative measures and law enforcement, trade in indigenous herbal remedies has flourished over the years (CUNNINGHAM, 1991). The reason for this, as Sherriffs (1995) suggests, is because traditional medicine practices are ingrained in the culture and traditions of the indigenous people of South Africa. Plants are carefully chosen for specific characteristics, their symbolic value and for the form in which they are to be taken (CUNNINGHAM, 1991). As the demand for medicinal plants is so highly species specific, trade in crinums, as with other traditional medicines, is likely to continue (CUNNINGHAM, 1991). Over collection of this rapidly dwindling reserve of indigenous flora may mean the loss of 50 000 species by the end of the century. In terms of economics, this represents a loss to the pharmaceutical industry of 25 prescription drugs with a market value of US$25 billion (SCOTT, 1993).

Fodder for animals

C. bulbispermum leaves are browsed by cattle (ROBERTS, 1990) while those of *Crinum delagoense*, including flowers and mature seed pods, are eaten by goats and cows. Reedbuck feed on (and reportedly enjoy) the flowers of *C. acuale* so that few seeds are produced (NICHOLS, 1984). This may be one reason why *Crinum* populations are so sparse and restricted in distribution (LEHMILLER, 1987).

Conservation for the future

The potential wealth of plants should be safeguarded for future generations (Biological Diversity Convention, Rio de Janeiro, June 1992) (HAMILTON, 1993). Plant-derived compounds, like galanthamine which is currently being tested in Alzheimer's disease therapy, are sometimes difficult to obtain and thus extremely expensive for clinical usage. Also, low yields recovered during organic synthesis make the process economically unattractive, with the result that plants still remain the best source of new drugs for clinical trials (SELLÉS *et al.*, 1997). It is possible that many of South Africa's indigenous plants will be future sources of food, medicines and other materials. In fact the potential value of our natural flora has been scarcely investigated (HALL *et al.*, 1980). Already, local medicinal uses of South African plants have shown promising leads for new curatives among a vast array of species (WATT & BREYER-BRANDWIJK, 1962). In a survey of the Southern African flora, it was reported that about 60% of the species from 11 plant families could show anti-cancer activities

(EDWARDS, 1976; cited by HALL *et al.*, 1980). Furthermore, many of the indigenous bulbs, including those from the Amaryllidaceae, have hardly been investigated from a horticultural point of view. So the preservation of all wild species is critical for the flower bulb industry (DE HERTOGH & LE NARD, 1993).

"One method of conserving our plants is through *successful* cultivation .... To bring most of the species into general cultivation depends on the few enthusiasts who are prepared to undertake the necessary research into correct cultural methods. These enthusiasts are hampered through lack of material with which to experiment and unless source material is more readily available it is feared that our bulbs will not survive the onslaught of modern living and will, fairly soon, be found only as dried herbarium specimens" (THOMAS, undated). It is the recommendation of the International Board for Plant Genetic Resources (IBPGR) that gene banks of medicinal plants be established worldwide and that heavily exploited species be domesticated as crops (SCOTT, 1993). Growing medicinal plants may also provide a major source of employment (STREAK, 1995). Indigenous plants are easy to care for and are fairly drought resistant. This makes them suitable for small-scale crop farming and community agriculture (STREAK, 1995). Already medicinal plant gardens are the focus of one community in The Valley of a Thousand Hills (BANG, 1996). "Careful husbanding of South Africa's medicinal plant resources would not only be sound conservation policy but good economic sense" (SCOTT, 1993).

Problems in the Amaryllidaceae

As a group, the Amaryllidaceae are unusual in terms of species conservation. And they "appear to be the most difficult with regards to maintaining a viable conservation collection" (KOOPOWITZ, 1986). This is because they possess several distinctive features that make them particularly vulnerable. "These are factors that arise from the biology of the organisms and (are) quite different from the man-made problems such as habitat destruction which are at the root of the problem. None is unique to the amaryllids, but it is the combination of so many that will make conservation difficult" (KOOPOWITZ, 1986). Biological factors that make conservation difficult include: long generation time, incompatibility factors, fleshy seeds which cannot be stored cryogenically in gene banks (KOOPOWITZ & KAYE, 1983), disease susceptibility and specialized cultural needs (KOOPOWITZ, 1986). Such problems have been encountered in South African members of the genus *Crinum*. Populations of *C. minimum*, for example, flower erratically and bulbs only produce one flower. This explains why populations are small and sparse (CRAIB, 1997). Germplasm which is maintained in field collections in the form of bulbs is subject to pests and diseases (DODDS, 1991). Furthermore, as the majority of species are tropical, they must be grown under protection in temperate climates. It is thus expensive to maintain an adequate gene pool (KOOPOWITZ, 1986).

"With the array of problems posed by the Amaryllidaceae, it's essential that those interested in this group and its immense horticultural potential conserve as much of this heritage as possible" (KOOPOWITZ, 1986).

Already the University of California Irvine (UCI) has a collection of South African bulbous and cormous plants, principally from the families Iridaceae and Liliaceae (KOOPOWITZ & KAYE, 1983) while Roodeplaat (Pretoria) has started a collection of the South African Amaryllids.

PROPAGATION

Cultivation of medicinal plants, as an alternative to collecting from the wild, was suggested 50 years ago especially for those plants that were rare and effective (GERSTNER, 1938; cited by CUNNINGHAM, 1990). To date, there has been no large scale cultivation, even although traditional medical practitioners generally accept cultivated material. There are two possible reasons for this: little institutional support and low market prices (CUNNINGHAM, 1990).

"The investigation and development of vegetative propagation techniques is a basic step in the domestication or ennoblement of wild plants. This may involve grafting, cuttings or tissue culture, and makes it possible to select for individuals showing high levels of desired characteristics" (CUNNINGHAM *et al.*, 1993). "Associated with this work is the development of a propagation system and infrastructure so that plants can be made available to users and to scientists" (CUNNINGHAM *et al.*, 1993).

Conventional propagation

Crinums may be grown from seed as well as from offsets (Figure 1.3). Although seeds are easier and more economical, Eliovson (1967) believes that "the easiest method of increasing bulbs is that of detaching the offsets or daughter bulbs that form beside the parent bulb (or corm) each season". *Crinum* hybrids such as those produced in the northern hemisphere e.g. Crinodonna, have a complex history and are not freely available. To preserve their clonal nature the bulbs are propagated vegetatively. There are, however, potential difficulties associated with these techniques.

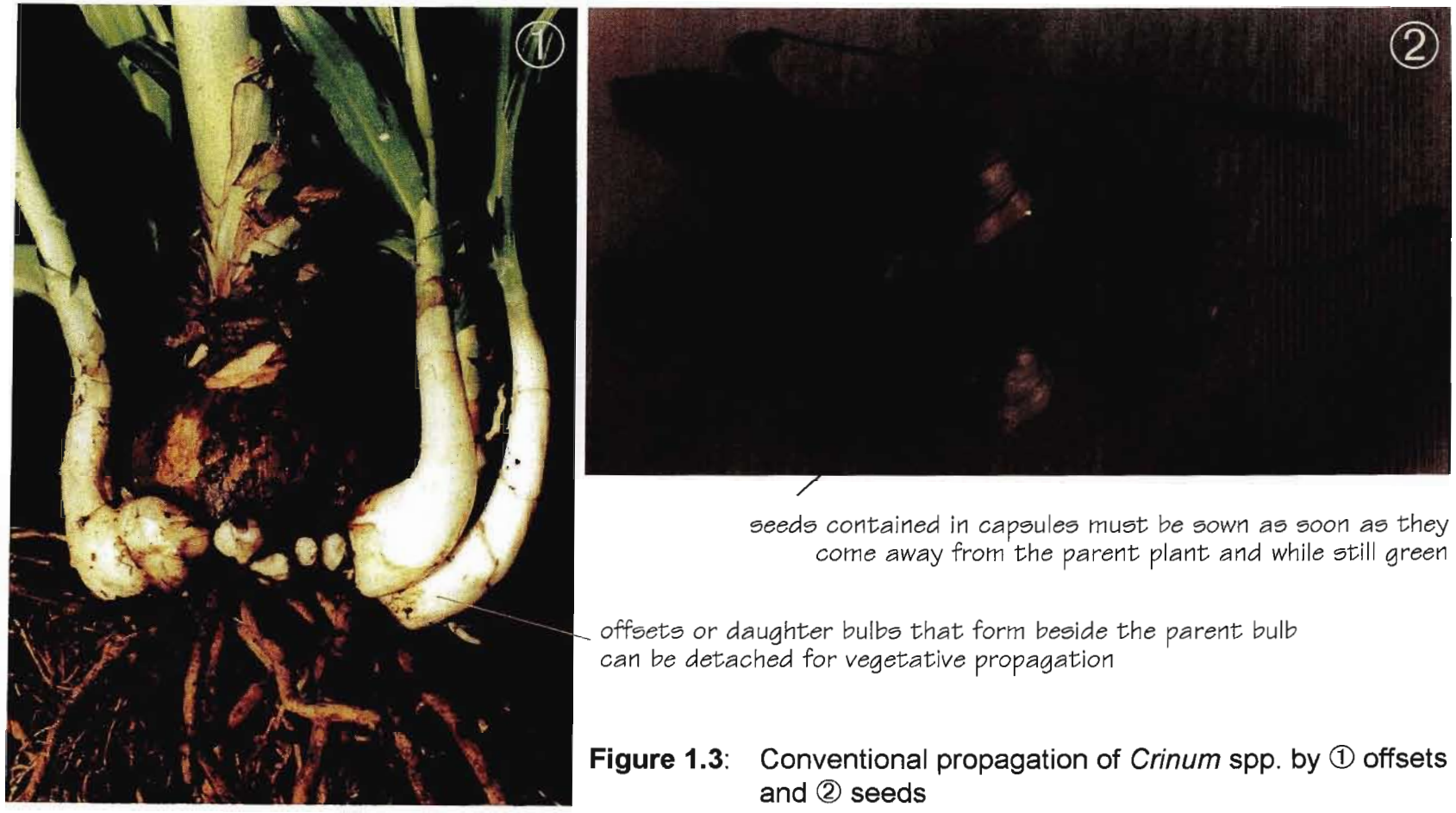


Figure 1.3: Conventional propagation of *Crinum* spp. by ① offsets and ② seeds

Seeds

Flowering of South African crinums is erratic as many species do not flower every year, but skip a season. Less than one quarter of *C. minimum* bulbs growing in populations in the Steilloopburg and Rustenburgkop areas flower each year (CRAIB, 1997). In some instances, less than 20% of the plants that do flower produce seed. These may abort before ripening or are eaten by amaryllis caterpillars and beetles (CRAIB & BLACKMORE, 1997). Also, the seeds often germinate on the plant. Although the seed coats are impervious to water, the water content of the seeds is high (ISAAC & McGILLIVRAY, 1965). This accounts for the peculiarity of the genus in that the seeds germinate readily without water and in the driest conditions (VERDOORN, 1973). The seeds must therefore be sown as soon as possible (i.e. as soon as they come away easily from the mother plants) and while still green (ELIOVSON, 1967). This is because their viability is short-lived (OLIVER,1990). As many amaryllids produce large fleshy seeds, they cannot be dried without killing the embryos (KOOPOWITZ, 1986). It may be possible to excise the embryos and process and freeze them using sterile tissue culture techniques. The process is, however, more involved and expensive than normal seed banking. Another problem associated with growing crinums from seed is that the juvenile phase of seedlings may last for several years. It is, therefore, possible that the seedlings may not be true to type. They may also be sterile (HANKS & REES, 1979). Furthermore, flowers are not produced for four years when propagation is from seed (ELIOVSON, 1980; BRYAN, 1989).

Offsets

Many species do not readily form offsets e.g. *C. bulbispermum* or only sporadically so (LEHMILLER, 1996). Large bulbs, in particular, multiply more slowly and that is why numerous offsets on *Crinum* bulbs are not often found (ELIOVSON, 1967). Some of the more desirable *Crinum* cultivars are slow and expensive to propagate using offsets; the better ones fetch as much as US$200 per bulb (ULRICH *et al.*, 1999). These are produced from axillary buds subtended within the leaves or scales of the bulbs. Only a few axillary buds are produced annually, and in some of the Amaryllidaceae they occur infrequently or rarely (HUSSEY, 1980). The offsets or bulbils have to be removed when still small to avoid damaging the fleshy roots. Mature bulbs are difficult to move because of their enormous size. They should be left undisturbed for many years as they perform best in a permanent position. The bulbs are sensitive to transplanting (HUXLEY *et al.*, 1992). That, says Eliovson (1980), "is their chief disadvantage". Some species will not flower successfully in the first season after being moved. They need a season to settle before flowering again (ELIOVSON, 1967).

Although the "twin-scale" technique may be the most productive method of vegetatively propagating certain bulbs, the number of bulbs that can be produced in a year is still limited (SEABROOK *et al.*, 1976). This is due, in part, to the susceptibility of the scales to drying out and rotting during incubation (HANKS & REES, 1979). The technique is also labour-intensive and good results are limited to a short period each year (HANKS *et al.*, 1986). A further disadvantage is that diseases are easily spread from the parent plant (KIM & DE HERTOGH, 1997).

Value of aseptic culture methods

Tissue culture is a worthwhile alternative to conventional techniques and has several advantages, namely: rapid multiplication; virus elimination and crop improvement (KIM & DE HERTOGH, 1997).

The Amaryllidaceae include many of the major flower crops. The principal horticultural cultivars are mostly complex hybrids that originated as single parent selections. Their exploitation and production is, therefore, dependent on vegetative propagation to ensure the genetic homogeneity of selected genotypes. Although bulbs and corms increase naturally by means of axillary buds, the rates of increase are too slow for new cultivars or disease-free stock to be introduced on a large scale (HUSSEY, 1982[a]). *In vitro* techniques provide a rapid means of producing such clones - even in monocotyledonous plants (HUSSEY, 1975; KROMER, 1985; cited by KUKULCZANKA & KROMER, 1988) - as well as recalcitrant species (KIM & DE HERTOGH, 1997). This accelerates propagation rates and therefore productivity (PRAKASH & PIERIK, 1993) and allows amaryllids to be grown commercially. This means that new bulbs can be introduced to diversify the choice of flowering plants available (LILIEN-KIPNIS *et al.*, 1994). There is the added advantage that they are disease-free (HUSSEY, 1982[a]). Micropropagation also allows for year round multiplication and the avoidance of long dormant seasons (YEOMAN, 1986; PRAKASH & PIERIK, 1993).

Plants can also be stored under disease-free conditions in suitable media at low temperatures. The advantages of this are that less space is required than that for dry storage of bulbs and the risks of losses through disease are minimized (BONNIER & VAN TUYL, 1997). Reserves of disease-free material are useful for breeding lines (BONNIER & VAN TUYL, 1997) or for species that tend to be quickly infected with the virus (HUSSEY, 1980). Programmed multiplication can then be undertaken according to the seasonal requirements of each species (HUSSEY, 1980).

Micropropagation has important practical applications in plant breeding, particularly in speeding up the breeding process (KRIKORIAN & KANN, 1986). Tissue culture techniques can be used to produce haploids (DODDS, 1991) and protoplasts. These are valuable in

producing novel hybrids, in overcoming incompatibility barriers and, in introducing new characteristics (FOWLER, 1986). Such variation can be exploited in the form of new cultivars (ABBOTT & ATKIN, 1987). Embryo rescue can also be used in difficult and incompatible crosses; both interspecific and intergeneric. This is particularly worthwhile in cases where sterility and incompatibility limits the number of selfings that can be made (DEBERGH, 1994).

One of the principal advantages of *in vitro* propagation is the in-built disease protection it affords. Viruses can be eliminated by meristem-tip culture and the disease-free condition maintained in successive cultures. By eliminating pathogens, not only is plant growth improved, but virus-free stocks of important bulb cultivars can be routinely introduced (HUSSEY, 1978). In addition, breeding for resistance can be achieved through genetic manipulation (YEOMAN, 1986).

Some plants are not easy to cultivate outside the country of origin. This is especially so for tropical species which have a narrow genetic tolerance. Many are important medicinal plants which need to be preserved. Tissue or shoot cultures provide an ideal approach to the propagation of species of high interest (FARNSWORTH & SOEJARTO, 1991). The Amaryllidaceae are important sources of pharmaceuticals and flavourings. However, they are present only in small quantities *in vivo*. Large amounts of material therefore are needed for isolation, structure elucidation, bioassays and clinical trials. This is even before synthesis is attempted. Furthermore, the accumulation of compounds is highly susceptible to geographical and environmental conditions. Axenic cultures can be used to produce large quantities of secondary metabolites and thereby provide a stable supply for chemical and clinical studies.

A common problem associated with phytochemical investigations is what happens to secondary metabolites during and following harvest. Ghosal *et al.* (1990; cited by Lewis, 1992) have shown that mechanical and insect injury stress causes hydrolysis of alkaloid conjugates which could also be accompanied by oxidative modification. According to Lewis (1992), enzymic modification undoubtedly takes place in air-dried plant material. This is the material most often used for extraction purposes as fresh material is less readily available (LEWIS, 1992). It is possible that protection against mechanical stress and enzymic hydrolysis, prior to extraction, may be achieved using *in vitro* grown plants. According to Bajaj *et al.* (1988), plant tissue culture can be used to discover new biochemicals as products may accumulate in the medium (BERGOÑÓN *et al.*, 1992) as well as in the cells. Furthermore, the possibility exists that these can be produced on a larger scale in bioreactors (BERGOÑÓN *et al.*, 1996)

Another advantage of tissue culture is that the culture conditions (both chemical and physical) can be controlled. As a result, optimal conditions for compound production can be ascertained e.g. galanthamine production in *Narcissus confusus* (SELLÉS *et al.*, 1997). Also, cells that show enhanced production can be selected and improved by inducing mutation or differentiation either chemically or through genetic engineering (MISAWA & NAKANISHI, 1988). Tissue culture also provides a unique system for studying secondary metabolite biosynthesis and the relationship between organogenesis and secondary production (BAJAJ *et al.*, 1988). Clonal material would be valuable in determining plant part and seasonal effects on alkaloid levels (ELGORASHI, 2000), for genetic effects were thought to be responsible for the intraspecific variation in studies using plants collected from the wild. *In vitro* systems are rapid and use less radioactivity and callus provides a source of continuous tissue for enzymes studies in alkaloid biosynthesis (SUHADOLNIK, 1964).

Thus tissue culture techniques can provide plant breeders and phytochemists with high quality material which is easily exchanged internationally (KIM & DE HERTOGH, 1997). In addition, micropropagated plants can be used to supply the herbal medicine trade, thereby alleviating the pressures on wild populations already compromised by uncontrolled and large-scale collections (HANNWEG *et al.*, 1996).

Chapter 2

TISSUE CULTURE OF *CRINUM* : A REVIEW

CRINUMS IN CULTURE

In 1988 Western Europe produced around 13 million ornamental bulbs and corms by micropropagation (PRAKASH & PIERIK, 1993). These included members of the Amaryllidaceae - a family which has a long history of micropropagation - particularly those of importance to the horticultural industry as ornamental flower bulbs e.g. *Amaryllis*, *Hippeastrum*, *Narcissus* and *Nerine* (HUSSEY, 1975, 1977, 1980, 1982; KRIKORIAN & KANN, 1986; VAN AARTRIJK & VAN DER LINDE, 1986). They are, however, not propagated as extensively as some of the lilies because the techniques are more difficult (VAN DER LINDE, 1992) and thus have had "minimal impact on the flower bulb industry" (KIM & DE HERTOGH, 1997).

A limited amount of work has been done on the tissue culture of *Crinum* species, as outlined below.

Indirect adventitious organogenesis of a *Crinum* species was reported by Yamada in 1963 (cited by KRIKORIAN & KANN, 1986). Callus from *Crinum* flower parts, especially ovaries, was capable of producing buds (these resembled bulbils) and plantlets.

Mullin (1970) reported the *in vitro* culture of a *Crinum* species using bulb-scale tissue in a modification of White's medium supplemented with coconut milk and 2,4-D. Growth was limited and the older tissues turned black. Although 2,4-D was replaced with NAA at 0.1 and 1.0 mg ℓ^{-1}, growth still remained limited in the first and second culture passages. Subsequent cultures were maintained and grew more vigorously on a complex opium poppy medium of Ranganathan *et al.* (1963)(cited by MULLIN, 1970).

In 1984, Crinodonna, an intergeneric hybrid, was amongst several hundred ornamental bulbs and corms cloned in the Netherlands. Although no details are available as to its propagation, Pierik (1987) recorded that 10, 000 plants were produced.

Kromer (1985) investigated, to a limited extent, the regenerative potential of *Crinum abyssinicum* bulbs. The potential for regeneration was not as marked as that for other plants investigated, particularly those in the families Liliaceae and Aracaceae. Rooting occurred readily and without special treatment.

In 1988, crinums were micropropagated commercially in the Netherlands (DEBERGH & ZIMMERMAN, 1991). Only 50 plants were produced, possibly because the crop was under development or being cloned for breeding purposes.

Slabbert *et al.* (1993) studied the regeneration and growth of bulblets from bulb-scale segments of *Crinum macowanii*. Shoots were induced on twin-scales using the medium of Murashige and Skoog (MS) (1962) supplemented with 0 - 20 mg ℓ^{-1} NAA and BA and on a Modified Murashige and Skoog (MMS) medium with 1,25 mg ℓ^{-1} ancymidol, 0.1 mg ℓ^{-1} NAA and 0.1 mg ℓ^{-1} kinetin (ANK). Bulblets of 5 mm or more were then split and subcultured on MMS and ANK or MS without growth regulators. Shoots regenerated on a wide range of BA and NAA concentrations. Increasing the BA concentration from 0 - 20 mg ℓ^{-1} had a stimulating effect on the total number of plantlets produced. A similar response was achieved in the absence of growth regulators. Bulbs responded differently to different treatments. According to Slabbert *et al.* (1993), this was associated with the age and size of the bulb and with the size of the explant. Ancymidol promoted the development of more than one offset on each split bulblet. The best regeneration of secondary plants was achieved on MMS and ANK; culturing the plantlets at 25°C was optimal for growth. Anatomical studies showed that shoots regenerated from between the scales on the basal plate and that they were multicellular in origin as both the epidermis and hypodermis were involved.

Slabbert (1993) also reported the regeneration of plantlets from flower stalk explants. However, factors such as the age of the inflorescence stalk and the time of the year, were found to restrict plantlet regeneration. Discs taken from the middle or basal parts of immature floral stems measuring 70 - 100 mm in length were subsequently shown to regenerate shoots (SLABBERT *et al.*, 1995). Shoot production was optimised by placing the discs with their proximinal ends on the medium and supplementing the medium with combinations of kinetin and IAA, kinetin and NAA or BA and 2,4-D (SLABBERT *et al.*, 1995). In most treatments, more shoots developed from discs incubated at 16 / 8 h light / dark than those placed in the dark. Rooting of these plantlets was achieved using sucrose at higher levels than those required for shoot induction. Since the plantlets were initiated adventitiously from the epidermis and subepidermal layers and not from intermediate callus, regeneration from floral stems could be used for multiplying bulbs of *C. macowanii* (SLABBERT *et al.*, 1995).

Parts of the inflorescence were also used for the *in vitro* propagation of *Crinum asiaticum* (MOHAMED, 1999).

One of the more desirable *Crinum* cultivars, 'Ellen Bosanquet', has been successfully micropropagated using tri-scales. Multiple shoot (8.4) and bulblet (2.8) formation were achieved on a MS based medium containing 35.5 µM BA. After four months the explants were removed to a hormone-free medium. Rooting and acclimatization took place *ex vitro* (ULRICH et al., 1999).

Ohshika and Ikeda (1994) isolated the gametophyte protoplasts from the embryo sac of *Crinum asiaticum* L. var. *japonicum* Baker using pectinase, hemicellulase, cellulase and pectolyase. The protoplasts were cultured in petri dishes containing liquid White's basal medium supplemented with sucrose at 0.3 M. The protoplasts were still viable after one month. However, no cell wall regeneration and cell division were observed.

Somatic embryos were obtained from the flower bud callus of *Crinum asiaticum* using 2,4-D, NAA and BA. Although callus also developed from bulb scales, this was not organogenic. Embryogenic potential was therefore limited to the flower buds but could be sustained for more than a year. Regenerated plantlets exhibited the normal chromosome number (MUJIB *et al.*, 1996).

FUTURE RESEARCH PERSPECTIVES

Apart from the fact that so little has been published on the micropropagation of *Crinum*, several aspects of the *in vitro* culture of *Crinum* species warrant special attention, as attested to in the literature. The technology also lends itself to long-term storage of germplasm for conservation purposes and alkaloid production for the pharmaceutical industry. Other areas for future research on ornamental geophytes are proposed by Le Nard and De Hertogh (1993), including the need to obtain cultivars with improved horticultural characteristics. How this might be achieved is outlined by Kim and De Hertogh (1997). Ziv (1997) believes that "several innovations still await further progress and advancement" of which automation; artificial seeds; the use of new plant growth regulators and molecular techniques for improved propagation; storage organ development; breeding and environmental protection, are singled out.

Micropropagation of *Crinum*: problems and prospects

An alternative explant source needs to be investigated because of the difficulty in decontaminating the bulbs. There are many reports for the regeneration of bulblets on floral stem explants. By using explants from the inflorescence stalk, the parent bulb would be protected.

Yamada (1963; cited by KRIKORIAN & KANN, 1986) showed that indirect regeneration from callus was possible. Whether this can be achieved for suspension cultures and protoplasts is uncertain and therefore needs to be investigated. Ohshika and Ikeda (1994) suggest that the media requirements for protoplast cell wall regeneration and division need to be critically studied.

Robbertse *et al.* (1983), in a study of the germination of *Crinum bulbispermum* and *Crinum macowanii*, report that light inhibits bulb formation in seedlings. It is therefore possible that light may have an effect on *in vitro* grown bulblets. The effect of light, other physical factors and nutrient requirements, as outlined by Van Aartrijk and Van der Linde (1986), need to be studied, since both bulb induction and growth are critical stages in the micropropagation process. Dormancy related requirements will also need to be satisfied prior to planting out, for little is known of the requirements for *Crinum* species.

There is also the possibility of using liquid-shake cultures for rapid growth and multiplication of plants *in vitro*. Rapid growth is achieved by stimulating the formation of proper meristems which is enhanced in liquid cultures as well as with gibberellin-synthesis inhibitors, like paclobutrazol and methyl-jasmonate (BOONEKAMP, 1997). Such a system would alleviate problems currently encountered in the micropropagation of geophytes, which in most instances is not profitable because it relies on "hand manipulation, small culture vessels and time consuming operations" (ZIV, 1997). Liquid cultures also facilitate scaling-up (ILAN *et al.*, 1995).

The application of tissue culture techniques to other *Crinum* species, particularly those important in the herbal medicine trade, will also need to be investigated. Ulrich *et al.* (1999) propose that micropropagation procedures that are developed for some of the more desirable cultivars may be adapted for other rarer species and for multiplying newer cultivars.

Potential for long-term *in vitro* storage

Seed storage meets the conservation needs of many crop plants (WITHERS, 1991). But for those plants that are vegetatively propagated or whose seeds are recalcitrant, *in vitro* techniques are a viable alternative. Among these are storage under conditions that induce slow growth, and cryopreservation. *Crinum* seeds germinate readily even without an external source of water (ISAAC & McGILLIVARY,1965). This is because the fleshy seeds have a

high water content. Like other amaryllidaceous species, they cannot be subjected to routine seed-banking but must be conserved *in vitro*. Such a germplasm collection requires less space than for bulb storage and reduces the risk of disease (TOWILL, 1988; cited by BONNIER & VAN TUYL, 1997). Lily germplasm, which is needed for breeding purposes, has been cryopreserved successfully (BOUMAM & DE KLERK, 1990; MATSUMOTO *et al.*, 1995; cited by BONNIER & VAN TUYL, 1997). Vegetative material can also be maintained for long periods by reducing the nutrient strength and increasing the concentration of sucrose (BONNIER & VAN TUYL, 1997). The induction of dormancy in geophytes, for long-term storage, has been reported for *Triteleia* (ILAN *et al.*, 1995).

Potential for alkaloid production

As the Amaryllidaceae are known to be rich in alkaloids, they were amongst the first plants to be studied *in vitro* to determine whether the alkaloids produced in the plant could also be produced in culture and if so, at what level (STABA, 1980). Yet, to date, the production of secondary metabolites in monocotyledonous bulbous plants is a field that has received little attention (BERGOÑÓN *et al.*, 1996). Members of the family used for the production of alkaloids *in vitro* include: *Hippeastrum* for Amaryllidaceae alkaloids and *Narcissus pseudonarcissus* for haemanthine. The production of Amaryllidaceae alkaloids is also being tried in other *Narcissus* species e.g. *Narcissus pallidulus* (BASTIDA *et al.*, 1992; cited by BERGOÑÓN *et al.*, 1992). But production and maintenance of callus containing secondary metabolites from monocotyledons is difficult (KRIKORIAN & KANN, 1986). The problem is circumvented in *Narcissus confusus* (BERGOÑÓN *et al.*, 1996) since galanthamine is produced in shoot clumps and is released into the liquid-shake medium. According to Bergoñón *et al.* (1996), this shows promise for scaling-up in bioreactors. Although galanthamine, which is increasingly used in the treatment of Alzheimer's disease (BERGOÑÓN *et al.*, 1996), can be extracted from plants of the Amaryllidaceae, high-yielding species are difficult to obtain. Furthermore, the steroselectivity of its reactions during organic synthesis and low yields make such a process economically unattractive (SÉLLES *et al.*, 1997).

RESEARCH OBJECTIVES

Aims

Of the three indigenous *Crinum* species that are recognized as being heavily exploited by the herbal medicine trade in KwaZulu Natal, and thus in need of conservation, only *Crinum macowanii* has been established in culture. Consequently, *Crinum moorei* was selected for further study. It has merit as an ornamental, worthy of development, and its medicinal value has yet to be investigated.

The aim of this project was to micropropagate *Crinum moorei* for the purposes of rapid and mass multiplication. This cannot be achieved using seeds or vegetative propagules. The propagules could, in turn, be used for the synthesis of secondary metabolites *in vitro*.

Production stages

Bulb micropropagation involves six stages: Stage 0 - selection and preparation of mother plants, Stage I - establishment of the aseptic culture, Stage II - multiplication of propagules, Stage III - obtaining, hardening and bulbing of plantlets, Stage IV - dormancy breaking of bulblets and Stage V - transfer to the natural environment (KIM & DE HERTOGH, 1997). Details can be found in the review of tissue culture of ornamental flowering bulbs (geophytes) by Kim and De Hertogh (1997).

Micropropagation may be achieved by enhanced axillary bud development, organogenesis and adventitious bud formation or by somatic embryogenesis (ZIV, 1997). Direct organogenesis was favoured over somatic embryogenesis since "uncertainties about the homogeneity of the end product and the relatively high costs of the (latter) approach make it unlikely to lead to a real revolution in the bulb-raising industry" (DE VROOMEN, 1995).

The present work focussed on: (1) the selection of explants and the establishment of an aseptic culture; (2) the production and multiplication of propagules from floral stems and twin-scales and (3) the transfer of bulblets to the natural environment. These will be discussed in greater detail in the chapters that follow. For a guide to the most important stages, the reader is referred to Figure 2.1.

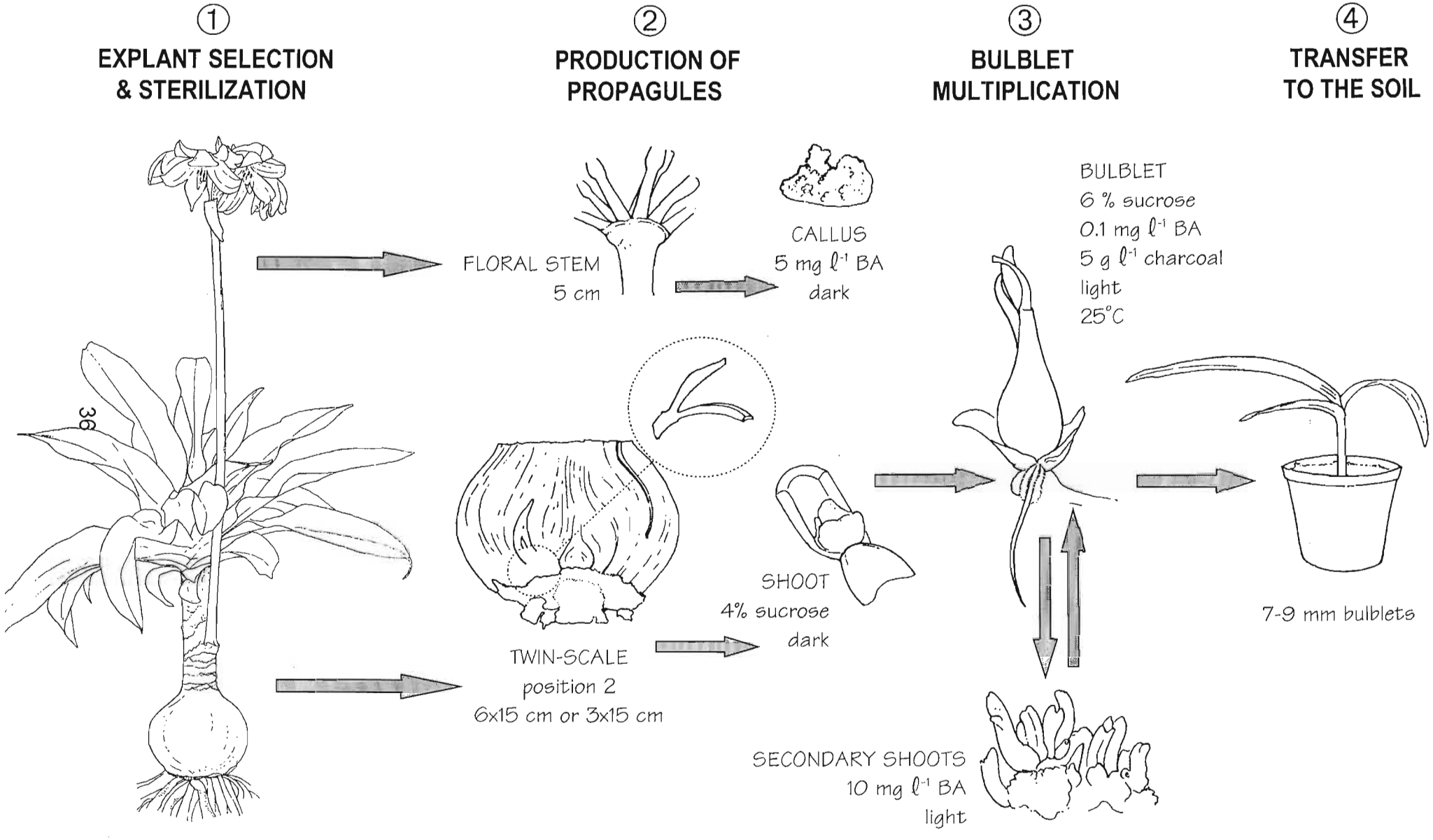


Figure 2.1: A summary of the production stages in the micropropagation of *C.moorei* (see text for details)

Chapter 3

EXPLANT SELECTION AND THE ESTABLISHMENT OF AN ASEPTIC CULTURE

Explant selection and sterilization

INTRODUCTION

Plant cells are totipotent and therefore retain their embryogenic competence. This means that explants from many different tissues have an inherent capacity to regenerate plantlets (VAN AARTRIJK & VAN DER LINDE, 1986). Meristematic structures will, however, form on specific parts of the explant and not on all explants with the same frequency (BOONEKAMP, 1997). Furthermore, tissues differ in their ability to undergo morphogenesis. Since cell types and endogenous factors play a key role in these first events it is necessary to select tissues or organs that respond well. These are usually meristematic regions such as shoot apices and axillary meristems in the axils of leaves. For bulbous plants, scales from the basal regions where they are joined to the basal plate; slices of young, unelongated stems and; buds that have already differentiated, have proved to be the most successful explants. While bulbs, leaves, inflorescence stems and ovaries of the Liliaceae all produce plantlets, only bulbs and inflorescence stems are productive in the Iridaceae and Amaryllidaceae (HUSSEY, 1978). Consequently, two *in vitro* systems are useful for the propagation of amaryllidaceous species, that is, regeneration from young floral stem explants and from twin-scales excised from bulbs. Of the two, proliferation of axillary shoots in the bulb scales is used almost exclusively (VAN AARTRIJK & VAN DER LINDE, 1986). This is despite the fact that the use of an underground perennating organ is frequently associated with heavy pathogen contamination. In addition to this, there is the problem of destructible bulb harvesting that eliminates any possibility of using the bulb for further vegetative propagation and horticultural evaluation (ZIV, 1997; ZIV & LILIEN-KIPNIS, 2000).

Inflorescence explants

While different tissues are used as explant sources in the micropropagation of bulbs, young inflorescence stems are reportedly the most consistently responsive tissues in propagating bulbs belonging to the Iridaceae, Liliaceae and Amaryllidcaeae (HUSSEY, 1975). Their regeneration potential has been shown to be superior to that of subterranean storage organs (ZIV & LILIEN-KIPNIS, 2000). Flower stalks of *Nerine sarniensis*, *Eucharis amazonica* (PIERIK, SPRENKELS & STEEGMANS, unpublished; cited by PIERIK & STEEGMANS, 1986) and *Hippeastrum* hybrids (PIERIK *et al.*, 1990) were found to be capable of regenerating adventitious bulblets, which suggests that "floral stems are likely to be an excellent source for *in vitro* propagation of most Amaryllidaceae" (PIERIK & STEEGMANS, 1986). Inflorescence explants of *Allium*, *Dichelostemma*, *Eucrosia*, *Haemanthus*, *Hyacinthus*, *Narcissus*, *Nerine* and *Ornithogalum* were also used to establish these bulbous species *in vitro* (ZIV & LILIEN-KIPNIS, 2000). Explants included the peduncle, pedicel-peduncle junction (from young, unopened inflorescences or older inflorescences) and pedicel tissue from an open capitulum (ZIV & LILIEN-KIPNIS, 2000). There are several advantages of using floral tissue rather than explants from the bulbs. Floral tissues are relatively pathogen-free (ZIV & LILIEN-KIPNIS, 2000) and can be excised from the plant without destroying the bulb. Also, plants at anthesis are at an ontogenetic stage where the plant breeder can assess the plant for desirable floral traits. Another advantage is that plants with a terminal mass of flowers, produced more or less synchronously, are equipped with a large number of potential meristems for propagation. These can be induced to develop vegetative buds i.e. revert to the vegetative phase, provided that they are excised at the appropriate developmental stage and, where necessary, cultured in media containing suitable growth regulators (ZIV & LILIEN-KIPNIS, 1997[b] and 2000). This presents fewer problems with respect to genetic abnormalities and no more than for plants propagated by conventional means (CRISP & WALKEY, 1974). However, not all bulbs flower every year (DE BRUYN *et al.*, 1992) and the possibility exists that off-types may be produced. Pierik and Steegmans (1986), for example, reported that bulblets formed adventitiously from callus on the cut surfaces of the peduncle explants of *Nerine bowdenii*. Since the *in vitro* grown plantlets had not yet flowered, they were unable to say with certainty whether they were true to type.

Young peduncles are known to be more successful in regenerating plantlets than mature floral stems, especially discs taken just below the pedicel-peduncle junction (PIERIK, 1991; JACOBS *et al.*, 1992; LILIEN-KIPNIS *et al.*, 1992; ZIV *et al.*, 1995).

Bulb explants

Although strong dominance of the main shoot prohibits axillary shoots from forming *in vivo,* bulb scales and pieces thereof, are known to freely produce shoots *in vitro*. However, among many species that perennate by subterranean storage organs, the regeneration potential *in vitro* may be species and genotype dependent, with the number of buds per isolated explant ranging from 2 - 25 (ZIV & LILIEN-KIPNIS, 2000). In the Amaryllidaceae, however, no bulblets form in the absence of the basal plate e.g. *Nerine* (PIERIK & IPPEL, 1977) and it is sometimes therefore necessary to include the basal plate when propagating bulbous plants (GEORGE, 1993). This is because small centres of meristematic cells occur where the leaves join the basal plate (THOMPSON, 1989). These are pre-existing axillary meristems (GROOTAARTS *et al.*, 1981) which may proliferate when induced by cutting the bulb (THOMPSON, 1989). Large numbers of bulblets of adventitious origin thus have the potential to be produced *in vitro* (GROOTAARTS *et al.*, 1981). Bulblets form in the axils of the scales from either existing meristems and/or adventitious meristems. Anatomical studies have shown that the adventitious shoots arise from the epidermis and hypodermis (HUSSEY, 1982[a]). As adventitious shoots are produced from such large multicellular meristems involving several layers of tissue at the base of the scale leaves, the potential for spontaneous mutation decreases (YEOMAN, 1986). "In fact the morphology of the bulbous monocotyledons with all shoot structures arising from the basal meristems may insure against the involvement of polysomatic tissue, provided only the basal tissues are used" (HUSSEY, 1978). Thus, the genetic stability of basal adventitious shoots may be similar to that of axillary shoots (HUSSEY, 1982[a]). It is important to exploit the natural mechanisms of axillary and adventitious shoot formation for long term multiplication and storage of selected genotypes in order to ensure maximum genetic stability but at the same time minimum loss of totipotency (HUSSEY, 1976).

Bulb scales are commonly used in the propagation of members of the Liliaceae, whereas twin-scales are used for the Amaryllidaceae. This follows the long-standing horticultural practice of propagating members of the Amaryllidaceae by twin-scaling. Known since 1935 and described in "The American Gardener's Book of Bulbs", twin-scaling involves cutting the parent bulb vertically into several wedge-shaped segments. These are further cut into smaller pieces each comprising two scales and a piece of the basal plate. This technique has been adopted for multiplying important cultivars *in vitro* on a much larger scale. Anatomical studies have shown that shoots are frequently initiated on the abaxial side of the bulb scales of amaryllidaceous species and that the vascular bundles of these shoots later connect with the vasculature of the outer scale (HUANG *et al.*, 1990). This development further supports the need to use twin-scales rather than single scales (HUANG *et al.*, 1990). Tri-scales have been used to micropropagate *Crinum* 'Ellen Bosanquet' (ULRICH *et al.*, 1999).

Mini-chips are also used as explants, especially when the addition of growth hormones has no stimulatory effect on the smaller twin-scales. They also produce more than one bulbil (SQUIRES & LANGTON, 1990). Chipping is a method derived from twin-scaling but is less critical and cruder. It has the advantage in that the technique can be mechanised and used on large batches of bulbs (REES, 1992).

Decontamination

Contamination is the most important reason for losses incurred in micropropagation systems. Bacteria and viruses, which remain latent for long periods of time, also result in reduced multiplication rates and virulence when the cultures are well-established (LEIFFERT & WAITES, 1994). For many vegetatively propagated plants it is essential that contamination is avoided. Debergh and Maene (1981) regard this as more important than inducing the first reaction.

Several methods of sterilization are commonly employed in the micropropagation of ornamental plants. Antibiotics, fungicides and other biocides may be incorporated into the medium, but as most broad spectrum biocides are not systemic, the internal plant tissues must be free of contaminants. It is therefore important to treat the stock plants. Disinfection of the plants is achieved by immersing the tissues either in sodium and / or calcium hypochlorite solutions or mercuric chloride. NaOCℓ is available as 5 - 10% of commercial bleach solutions. The concentration and duration of treatment must be chosen so as to minimize explant damage (DODDS, 1991). A wetting agent is usually added to aid penetration. Sometimes 70 - 90% ethanol is used as a pretreatment to allow better penetration or to act as a surfactant (DODDS, 1991). Alternatively, mercuric chloride is used when the contaminants are fungi and the tissue is sensitive to the hypochlorite treatments. Mercuric chloride is, however, highly toxic and its persistence may reduce tissue viability. A higher percentage decontamination may be achieved by using mercuric chloride in conjunction with a systemic fungicide such as benomyl (LEIFFERT & WAITES, 1994). Benzimidazole fungicides affect cytokinesis by interfering with fungal microtubules (ROY & FANTES, 1982; cited by SHIELDS *et al.*, 1984).

High contamination rates are often observed in cultures initiated from soil-borne organs e.g. bulbs, rhizomes and stolons (KUNNEMAN & ALBERS, 1989; cited by HOL & VAN DER LINDE, 1992) despite submersion in hypochlorite solutions or mercuric chloride. Since storage organs are used for vegetative propagation and are, for many bulbous plants, the final product in micropropagation schemes, pathogens are perpetuated with each multiplication cycle. The accumulation of pathogens thus significantly limits production. To remedy the problem, hot water is sometimes used as a post-harvest tissue culture pretreatment to remove endogenous

pathogens. As many pathogens are more heat sensitive than their host tissues (GILAD & BOROCHOV, 1993) elevated temperatures are effective in inactivating and thereby removing many viruses and some systemic bacterial diseases (SIVANESAN & WALLER, 1986; cited by GEORGE, 1993). A further advantage of a temperature treatment is that there are no chemical residues (LANGENS-GERRITS *et al.*, 1998). Newton *et al.* (1933; cited by HANKS, 1993) maintain that if it is not used, other chemicals are unable to penetrate between the scales unless these are applied under vacuum. The use of heat to "cure" plants of diseases and pests during *in vivo* propagation was first recorded in 1888 for oats and barley. In 1918 it was used to treat *Narcissus* bulbs with nematode disease. Today, it is a common treatment in agricultural practices for diseased bulbs and tubers such as *Iris*, lily, *Gladiolus*, tulip and hyacinth (HOL & VAN DER LINDE, 1992) and seeds (LANGENS-GERRITS *et al.*,1998). In general, bulbs and tubers are placed in hot water or air at 50 - 52°C and left for periods of 10 - 30 minutes (GEORGE, 1993). A hot water treatment of 35 - 45°C (HWT) was found to reduce contamination to 5% in tissue cultured *Narcissus* bulbs (HOL & VAN DER LINDE, 1992). It is especially useful when the contamination frequency of the outer, older parts of the bulb is high (SQUIRES & LANGTON, 1990).

MATERIALS AND METHODS

Collection and cultivation of parent bulbs

Crinum moorei plants were acquired from the National Botanical Gardens, Pietermaritzburg and from Mrs Verity of the farm "Woodwind" in Balgowan. As summer growers, they prefer a heavy, rich soil. Good drainage, is however, a requirement for pot culture. The bottom of the pot was therefore covered by pieces of bark and an intermediate layer of well-decomposed compost. A mixture of acid sand (grain size 0.5 mm), loam and compost is recommended for summer-growing bulbs (DU PLESSIS & DUNCAN, 1989), and so *Crinum* bulbs were placed in a pot mixture comprising four parts sieved garden compost, two parts fine bark chips and one part industrial sand, finely sieved. Mature bulbs vary in size but are known to attain a diameter of up to 19 cm (VERDOORN, 1973). The roots are fleshy and perennial. If damaged, leaf regeneration is impaired and inflorescences may not be produced in the following growing season. The bulbs are therefore best left undisturbed (DU PLESSIS & DUNCAN, 1989). For this reason, *Crinum* plants were planted in large 24 - 30 cm diameter pots with the tunicated, neck region above soil level. Shallow planting is recommended to allow for the development of the rootstock. As it is a forest species (from the eastern Cape), which flourishes in dense or partial shade, the pots were placed under shadecloth and watered by an overhead irrigation system. The plants are heavy feeders and require regular feeding with an organic fertilizer such as Kelpak.

The amaryllis borer (*Brithys pancratii*) frequently gorges itself on the leaves and floral parts. Plants were therefore carefully monitored and affected parts removed or treated with a recommended pesticide such as Bexadust.

During the flowering period, November to March, inflorescences were obtained from plants growing in the National Botanical Gardens in Pietermaritzburg and in the gardens of the Botany Department.

Explants

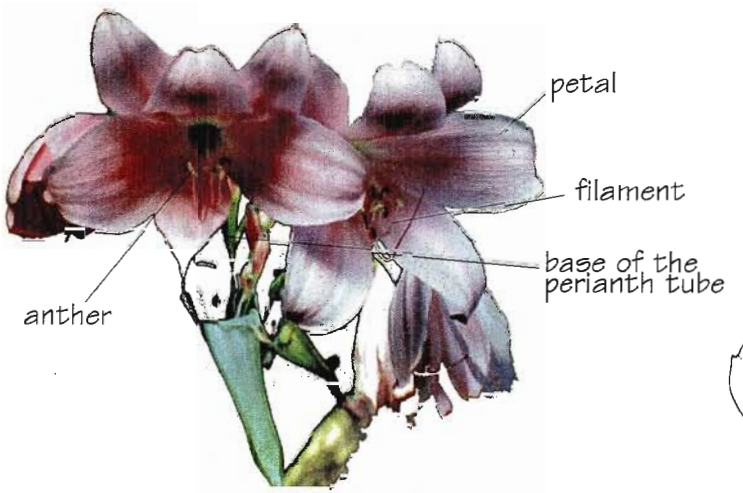
Explants from both the aerial shoot and soil-borne storage organ were selected (Figure 3.1).

Inflorescences were collected in December and January. Floral explants included:

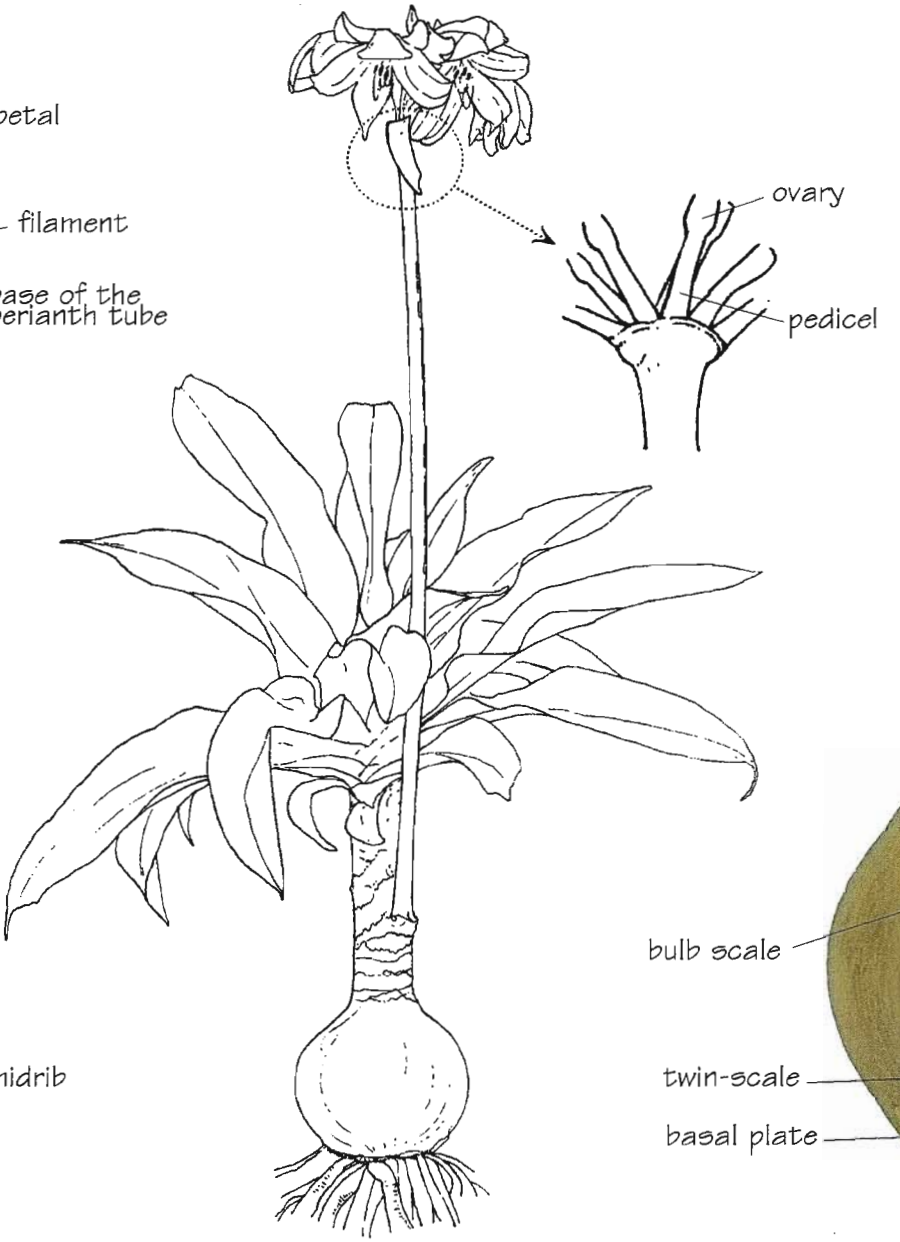
- **Anthers** - excised from 10 cm buds where the petals had not yet opened. At this stage the anthers were cream to grey in colour.
- **Filaments** - excised from 10 cm unopened buds. The explants measured 15 mm in length.
- **Petals** - segments, approximately 1 cm^2 were cut from the thickened midrib region of flowers in the mature bud stage.
- **Base of the perianth tube** - circular, 2 mm thick portions.
- **Ovaries**- circular, 2 mm thick sections
- **Pedicels** - circular, 2 mm thick sections
- **Peduncles** - 16 - 32 cm in length were removed from the plant before anthesis. Circular, 2 mm thick sections were used.

Leaves were used at the start of the new growth season (April). These had not yet fully expanded. Sections were taken from the midrib region of the basal part of the leaf.

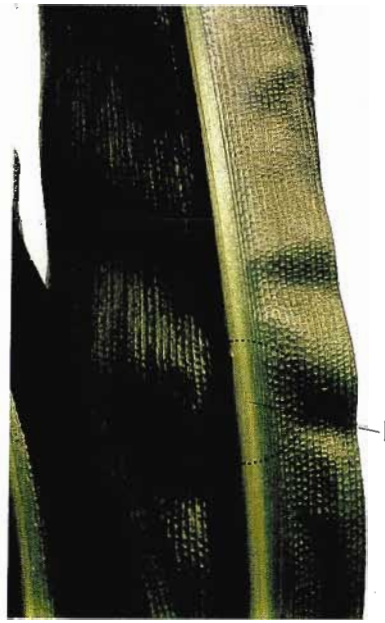
Twin-scales and bulb scales were excised either from young offsets that had been removed from the parent bulb or from mature, flowering-size bulbs. The dry, outer scales, roots and tunica were first removed before cutting the bulbs longitudinally into two symmetrical parts. Each half was further divided into four radial segments of equal size. These were used to prepare the twin-scales and bulb scales (Figure 3.2). The twin-scales included two adjacent bulb scales conjoined to a 3 mm portion of the basal plate. The explants measured 15 mm in length and 5 mm in diameter. Individual bulb scales of the same size were excised from the lower half of the bulb. The basal plate was not included. Excision took place at various times during the active growing period.



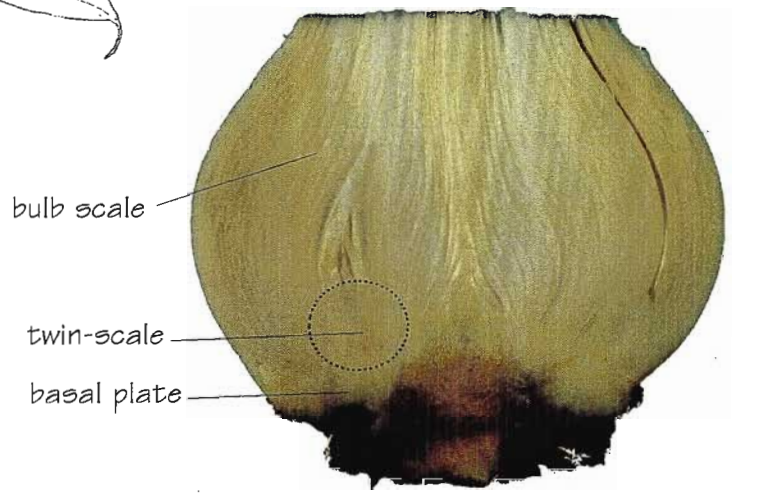
Explants from the inflorescence



Discs excised from the peduncle

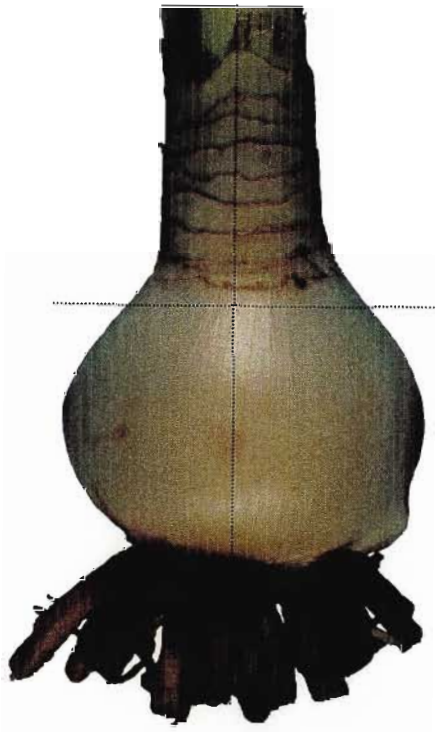


Leaf explants from the midrib



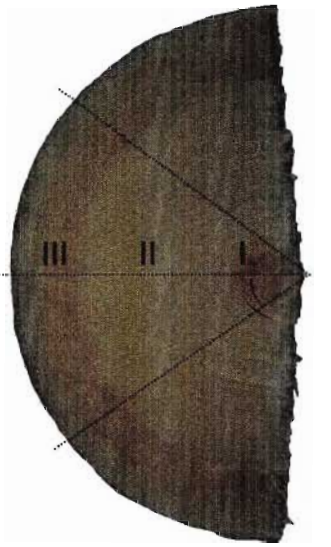
Explants from the bulb

Figure 3.1: Source of explants from *Crinum moorei*

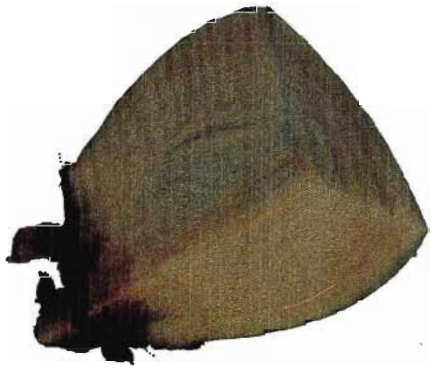


①

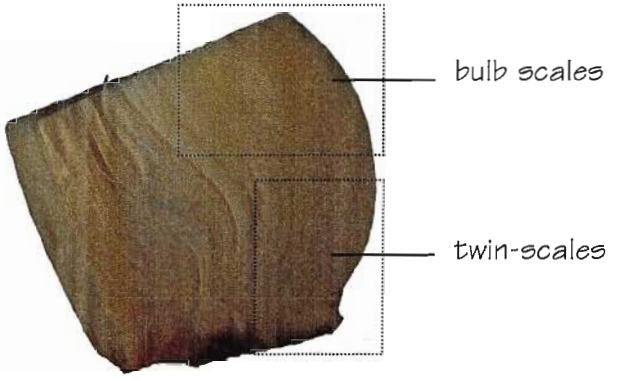
②



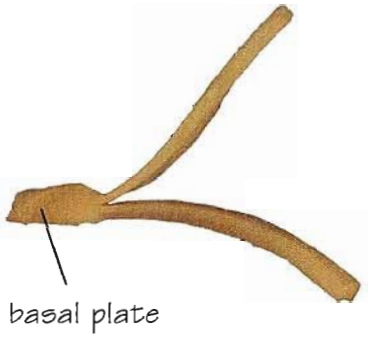
③



④



⑤



⑥

Figure 3.2: Preparation of bulb explants

1. Bulbs cut longitudinally into two symmetrical parts
2. Half-cut bulb showing position of inner (I), middle (II) and outer (III) scales
3. Division into four, equal-sized segments
4. Roots and old basal tissue removed
5. Excision of twin-scales and bulb scales
6. Twin-scale consisting of two adjacent scales joined by the basal plate

Steps 5 and 6 performed under aseptic conditions.

Decontamination

Preparation of the plant material

Plant parts were thoroughly rinsed in running water after discarding damaged and necrotic tissues. Where bulbs served as the explant source, roots were trimmed to the basal plate and the outer papery scales and upper half, comprising bulb scales, removed. The bulb was then cut into several wedge-shaped segments. The older, yellowing basal plate was removed together with the remnants of roots. The bulb material was further rinsed in water containing Tween 20™ and then placed in 0.2% Benlate for 5 - 10 minutes, unless otherwise stated.

Decontamination of inflorescences and leaves

NaOCℓ, commercially available as Jik™, was used as the sterilant. Concentrations of 1, 2 and 3.5% were tested for time periods lasting between 5 and 20 minutes. Tween 20 was used as a surfactant in the sterilant. Decontamination was followed by three sterile distilled water washes of 5, 10 and 10 minutes each.

Decontamination of bulbs

Both NaOCℓ and mercuric chloride were tested as sterilants for the bulbs. NaOCℓ was used as 2 and 3.5% solutions. The duration of treatments ranged from 5 to 20 minutes. This was extended to 40 minutes when full-strength Jik i.e. 3.5% was used. Mercuric chloride ($HgC\ell_2$) was used at a concentration of 0.1%. Explants were immersed in the solution for 2, 5, 7 or 10 minutes without presterilizing in Benlate™. Tween 20 was added to the sterilant to improve wettability during agitation. At the end of the decontamination period, the plant material was subjected to a series of three sterile distilled water rinses of 5 - 10 minutes.

Many potential contaminants are endogenous and therefore survive surface sterilization with most germicidal agents. This is especially so for bulbous geophytes where microbes find their way into small crevices such as those between bulb scales. Their locality can be determined by dissecting explants from various parts of the bulb. In this instance, twin-scales of *Crium moorei* were divided into three groups: inner, middle and outer (representing their position in the parent bulb) (Figure 3.2) and decontaminated in 3.5% NaOCℓ for 30 - 35 minutes. Various presterilization treatments were tested for their efficacy in removing these contaminants and wetting the tissues to allow for better penetration of the sterilant. Ethanol and Sporekill™ were used as chemical pretreatments. The effectiveness of Hot Water Therapy (HWT) was also investigated. Bulb segments were dipped in 70% ethanol for 30 seconds, 1 minute or 2 minutes, after soaking in Benlate, or Sporekill which was prepared

as 1 and 2% solutions. For the HWT, bulb segments were placed in 250 mℓ Schott bottles in water baths set at 50°C and 54°C. The HWT's were of 15 and 30 minutes duration at each temperature. Thereafter the bulbs were placed in 0.2% Benlate for 10 minutes and sterilized in 3.5% NaOCℓ for 35 minutes.

Aseptic culture methods

After decontamination, further work was performed behind a lit spirit burner. Explants were dissected in sterile petri dishes and tissue damaged during the decontamination operations removed. Following excision of the plant tissue, explants were inoculated on to the culture medium and the mouth of the culture vessel flamed. The vials were then sealed with parafilm or Cling Wrap™ to reduce infection by air-borne contaminants.

Statistical analysis of data

Where appropriate, results in this, and subsequent chapters, were subjected to a one way analysis of variance (ANOVA) or *t* - Test (Minitab Inc., 1995) to assess differences in the mean values of the variables tested ($P < 0.05$). Differences are denoted in the tables and figures by different letters.

RESULTS

Decontamination of inflorescences and leaves

The decontamination of aerial organs was effective over a wide range of treatments tested (Table 3.1). A 1% solution of NaOCℓ for 20 minutes resulted in 70 - 100 % decontamination of anther, filament, petal, ovary, pedicel, peduncle and leaf explants.

Table 3.1: Percentage inflorescence and leaf explants successfully decontaminated and viable

Inflorescence and Leaf explants	Sterilant concentration (%) and duration of treatment (minutes)											
	1% NaOCℓ				2% NaOCℓ				3.5% NaOCℓ			
	5	10	15	20	5	10	15	20	5	10	15	20
Anther	80	100	30	100	0	10	60	100	-	-	-	-
Filament	90	90	80	100	0	0	90	90	-	-	-	-
Petal	50	10	30	70	0	0	60	60	-	-	-	-
Base of perianth tube	90	100	100	90	80	100	100	100	100	100	100	100
Ovary	100	100	100	100	80	80	100	100	90	100	10	100
Pedicel	100	100	100	100	50	80	80	90	90	90	20	100
Peduncle	100	90	100	90	0	0	50	30	100	20	0	80
Leaf	90	60	90	100	100	50	80	80	90	100	90	90

Decontamination of bulbs

The bulbs proved more difficult to decontaminate with NaOCℓ (Table 3.2). At a concentration of 2%, very few explants were decontaminated. When placed in 3.5% NaOCℓ for 5, 10 or 15 minutes, contamination of the explants was also widespread. However, subjecting the tissues to longer time periods in concentrated NaOCℓ resulted in 56 - 70% decontamination.

Although 0.1% $HgCℓ_2$ decontaminated the bulb explants, the toxicity of this sterilant resulted in the death of the explants (Table 3.2).

Table 3.2: Percentage bulb explants decontaminated and viable

Bulb explants	Sterilant concentration (%) and duration of treatment (minutes)															
	2% NaOCℓ				3.5% NaOCℓ								0.1% $HgCℓ_2$			
	5	10	15	20	5	10	15	20	25	30	35	40	2	5	7	10
Bulb scales	20	60	0	0	0	10	0	20	-	-	-	-	0	0	0	0
Twin-scales	0	10	0	0	0	10	0	70	70	56	68	0	0	0	0	0

The persistence of contaminants was undoubtedly due to their endogenous habit. Tests showed that while the inner and middle twin-scales were relatively free of micro-organisms, most pathogens infected the outer, older twin-scales (Table 3.3).

Table 3.3: Percentage twin-scales decontaminated from different positions within the parent bulb

Bulb explants: Twin-scale position	Sterilant (3.5% NaOCℓ for 30 - 35 minutes)
Inner (Position I)	83
Middle (Position II)	58
Outer (Position III)	30

The use of a presterilant reduced the high incidence of bacterial and fungal contaminants in the medium to 8%. A combination of Benlate and ethanol (1 minute) or 1% Sporekill and ethanol (1 minute) sterilized 92% of the explants. All explants produced shoots following the latter treatment. The mean number of shoots per explant was also higher at 2.55 compared to 2.35 for the Benlate and ethanol treatment although this was not significantly different (Table 3.4).

Table 3.4: Percentage twin-scale explants decontaminated and viable after chemical presterilization

Twin-scale explants	Presterilant					
	0.2% Benlate (10 mins) CONTROL	0.2% Benlate (10 mins) 70% Ethanol (30 secs)	0.2% Benlate (10 mins) 70% Ethanol (1 min)	0.2% Benlate (10 mins) 70% Ethanol (2 mins)	1% Sporekill (30 mins) 70% Ethanol (1 min)	2% Sporekill (30 mins) 70% Ethanol (1 min)
Decontamination & survival (%)	84	30	92	84	92	88
Shooting (%)	90	73	91	74	100	91
Mean number of shoots/explant	1.53^{b}	1.43^{ab}	2.35^{ab}	1.22^{b}	2.55^{a}	2.0^{ab}

Different letters show significant differences between treatments at the 5% level (ANOVA).

All twin-scale explants that were heat-treated at 50°C for 15 minutes were sterile. Shoots were produced in 85% of the explants. Survival was, however, poor when the bulbs were heat-treated for 30 minutes and at a higher temperature of 54°C (Table 3.5). Compared to the control treatment, fewer explants developed shoots at 50°C. The mean number of shoots per explant was also lower but not significantly so (Table 3.5).

Table 3.5: Percentage twin-scale explants decontaminated and viable after HWT

Twin-scale explants	Presterilant				
	0.2% Benlate (10 mins) CONTROL	50°C (15 mins) 0.2% Benlate (10 mins)	50°C (30 mins) 0.2% Benlate (10 mins)	54°C (15 mins) 0.2% Benlate (10 mins)	54°C (30 mins) 0.2% Benlate (10 mins)
Decontamination & survival (%)	5	100	35	0	0
Shooting (%)	100	85	29	0	0
Mean number of shoots/explant	2.0[a]	1.53[a]	1.0[a]	0[a]	0[a]

Different letters show significant differences between treatments at the 5% level (ANOVA).

DISCUSSION

Decontamination of inflorescences and leaves

Inflorescences and leaves of healthy stock plants were successfully decontaminated using NaOCℓ. This is commonly used in the aseptic culture of herbaceous plants as it oxidizes superficial contaminants without being toxic to the plant tissues. As a general rule, a 1% solution of NaOCℓ can be used to disinfect tissues from both the inflorescences and leaves of *C. moorei.*

Decontamination of bulbs

NaOCℓ was not as effective in decontaminating the bulbs. This was because systemic pathogens were located in the older, outer bulb scales and were, therefore, not affected by surface-disinfestation procedures. Storage tissues are difficult to free of contaminants; bulbs more so than corms (HUSSEY, 1975) because their open structure allows micro-organisms to move in between the scales. The outer dried sheathing leaves may also harbour mycorrhizae. Bulbs of *Crinum asiaticum,* for example, were reported to be heavily infested with VA mycorrhizae (IQBAL & BAREEN, 1986). While undissociated HOCℓ can penetrate easily into the bacterial cell, penetration is not as easily effected when the negative OCℓ (hypochlorite) ion is used. In alkaline solutions, these dissociated ions are in abundance, making it more difficult for chlorine to kill germs at a pH greater than 7. In other instances, though, a hypochlorite solution (0.525%) was effective in decontaminating bulb chips of *Crinum* 'Ellen Bosanquet' when the immersion time was 1 hour (ULRICH *et al.*, 1999). A double decontamination procedure was employed whereby the tri-scales were rinsed in a weaker hypochlorite solution (0.263%) for 5 minutes after excision (ULRICH *et al.*, 1999).

None of the bulbs treated with mercuric chloride survived sterilization. Heavy metal ions, such as those present in mercuric chloride, are known to be toxic to plant tissues. This would account for the loss in tissue viability and absence of shoot production. However, the sterilant has been used successfully in the sterilization of *Cyrtanthus* bulbs (McALISTER *et al.*, 1998[b]) although there appeared to be no consistency in the degree of decontamination achieved.

There was a notable improvement in the decontamination rate following chemical and hot-water pretreatments. The effectiveness of Benlate - a carbamate-based fungicide - can be attributed to its systemic action i.e. it is taken up and translocated by plants (SOLEL *et al.*, cited by THURSTON *et al.*, 1979); inhibition of fungal cytokinesis (ROY & FANTES, 1982; cited by SHIELDS *et al.*, 1984) and ability to repress mycorrhiza development (HONG, 1976; cited by THURSTON *et al.*, 1979; KRITZINGER *et al.*, 1998). Unlike some other antifungal agents, benomyl shows a broad spectrum of fungicidal activity and non-toxicity at high concentrations (when dissolved by boiling or autoclaving). This makes it ideal for controlling and preventing fungal contamination (HAUPTMANN *et al.*, 1985), and more especially since it is reported to have no inhibitory effect on tissue cultures (KRITZINGER *et al.*, 1998). Other perennating organs, such as the rhizomes of *Zantedeschia aethiopica*, have been satisfactorily disinfested using a fungicide pretreatment, although this was of longer duration (KRITZINGER *et al.*, 1998). The fungicide used was Dithane which, like benomyl, contains carbamate as an active ingredient. However, other workers generally avoid the use of benomyl in the disinfestation of bulbs. It is reported to have "an unexpected effect" on gladiolus corms (FORSBERG, 1969; cited by PIERIK & RUIBING, 1973) and its application was avoided in the treatment of hyacinth bulbs and the twin-scales of *Narcissus* because of its adverse effect on rooting (HANKS & REES, 1979). It is also thought to reduce bulbil initiation (HANKS & REES, 1979). In other species, benomyl may either promote or inhibit root and shoot growth, depending on the concentration used (SHIELDS *et al.*, 1984). Apart from these known side effects, benomyl-resistant isolates of *Penicillium* species and *Botrytis cinerea* have been isolated from treated tissues of *Narcissus* species following the required incubation period (HANKS & REES, 1979), suggesting the need for more effective fungicides.

The function of alcohols is to remove waxes from the surfaces of plant parts, thereby permitting other sterilants to penetrate the tissues. They also exhibit germicidal action (GEORGE, 1993). Their dual action would account for the decrease in contamination of *Crinum moorei* twin-scales following sterilization in NaOCℓ. The bulbs of several other ornamental species were sterilized based on treatment with alcohol at various concentrations for 30 -120 seconds (VIDOR, 1997) with or without further treatment in $HgC\ell_2$, hot water at 54°C or the fungicide miconazole.

A HWT at 50°C for 15 minutes reduced contamination to 0% in *C. moorei* twin-scales. The most successful sterilization method for *Crinum* 'Ellen Bosanquet' involved soaking the bulbs in hot (50°C) tap water for 1 hour prior to treatment in sodium hypochlorite (ULRICH *et al.*, 1999). A 35 - 55% reduction in contamination was reported for twin-scale cultures of several *Narcissus* cultivars (HOL & VAN DER LINDE, 1992) although a temperature of 50°C was found to be less effective. Since tubers are reported to have a relatively low thermal conductivity (GILAD & BOROCHOV, 1993), entire bulbs would require high temperatures and several hours of hot water therapy to effectively destroy systemic pathogens. A higher temperature of 54°C was, therefore, more appropriate in reducing contamination in intact *Narcissus* (HOL & VAN DER LINDE, 1992) and other ornamental bulbs (VIDOR, 1997) compared to those of *Crinum moorei* where wedge-shaped segments, rather than whole bulbs were used. Furthermore, different pathogens have different heat sensitivities (LANGENS-GERRITS *et al.*, 1998) which might explain why different host tissues need higher or lower temperature treatments. The reason why HWT was effective is possibly because it allowed better penetration of chemical sterilants between the scales. *Narcissus* bulbs that are heat treated are placed in HWT tanks with added fungicides. Benzimidazole fungicides are most often used (HANKS, 1993). Compared to the chemical pretreatments, initial bud growth of heat-treated *C. moorei* bulbs was impaired. Hot water reduced regeneration in the bulbs of several other ornamental species, namely *Hyacinthus amethystina*, *Tulipa gesneriana*, *Iris hollandica* and *Narcissus* hybrids (VIDOR, 1997) but was nevertheless recommended as part of the aseptic regime for cleaning the bulbs. Tuber injury and reduced growth were also reported for *Liatris* tubers following heat therapy (GILAD & BOROCHOV, 1993). The possibility that membrane protein thiols are oxidized during HWT may account for tissue damage during heat stress (GILAD & BOROCHOV, 1993). If this is the case, Gilad and Borochov advocate the use of suitable antioxidants to decrease tuber sensitivity to heat stress. Because bulbs are submerged during HWT's, a deficiency in oxygen may lead to the production of acetaldehyde and ethanol. The release of these compounds, in turn, causes tissue deterioration and even inhibition of shoot proliferation and rooting in some cultures (LANGENS-GERRITS *et al.*, 1998).

CONCLUSIONS

The most effective means of decontaminating *C. moorei* bulbs, while at the same time maintaining tissue viability, was to soak them in 1% Sporekill for 30 minutes; dip them in 70% ethanol for one minute and then decontaminate the segments in 3.5% NaOCℓ for 30 minutes (Figure 3.3). By selecting only the inner twin-scales, fewer explants would be contaminated. The disadvantage of this is that bulbs would yield fewer usable explants.

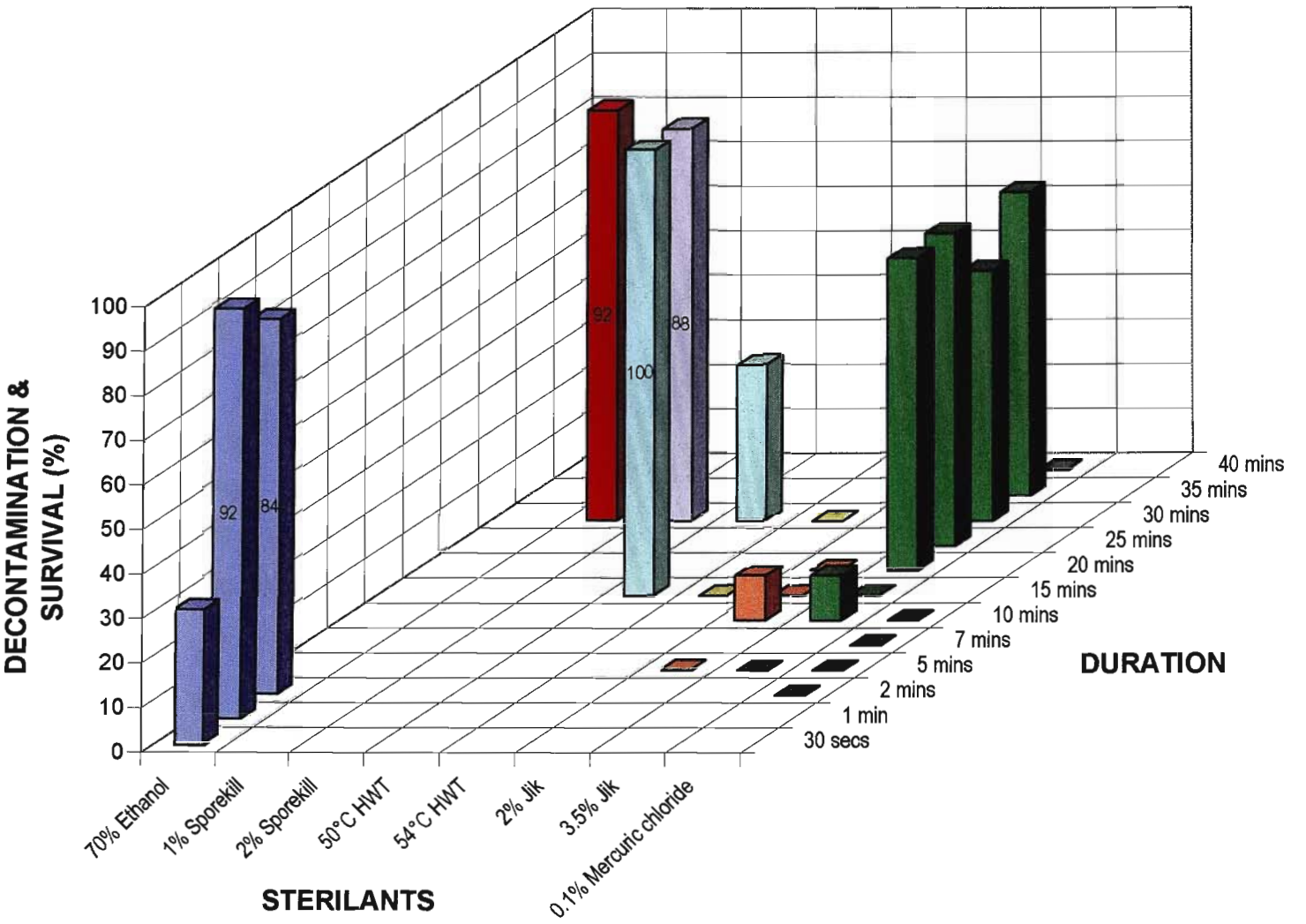


Figure 3.3: The effectiveness of different sterilization treatments in decontaminating the bulbs

The induction of growth & morphogenesis

INTRODUCTION

Success at stage one of any micropropagation scheme depends on the establishment of an aseptic culture followed by the growth of the explant (GEORGE, 1993). However regenerative an explant may be, regeneration almost always requires a nutrient medium containing salts, sugar, some vitamins and hormonal supplements (VAN AARTRIJK & VAN DER LINDE, 1986); the most critical components being auxin and cytokinin (MURASHIGE, 1974). Endogenous levels of both auxin and cytokinin may vary considerably between species and even between organs of the same plant. Furthermore, the requirement for exogenous plant growth regulators may be influenced by the duration and temperature of storage, which is of particular importance in the commercial practice of bulb production. The "Skoog-Miller" model, therefore, does not hold true for all species and so levels of growth-promoting substances must, in practice, be empirically determined.

Low concentrations of auxin ($<10^{-6}$M) increase the induction of cell divisions, leading to shoot production in many bulbous crops (VAN AARTRIJK & VAN DER LINDE, 1986). For example, low levels of NAA stimulated the formation of buds in *Hippeastrum hybridum* bulb scales (MII *et al.*, 1974). The type of auxin used is also critical. For monocotyledons, in general, NAA is more effective than 2,4-D (HUGHES, 1981). High auxin levels also result in callus production from ovaries e.g. *Narcissus* cultivars (SEABROOK *et al.*, 1976) and flower stalks e.g. *Narcissus* cultivars (HOSOKI & ASAHIRA, 1980).

The promotive effect of cytokinins on shoot production has been reported for many species of monocotyledons where apical dominance normally inhibits shooting (HUSSEY, 1976[a]). Compared to the Iridaceae, amaryllidaceous species require higher cytokinin levels to promote branching (HUSSEY, 1976[a]). Adventitious bud formation in *Narcissus* cultivars was stimulated (HUSSEY, 1976[a]) using flower stalks and leaves (HOSOKI & ASAHIRA, 1980). Where bulbs are used as explant sources, cytokinins are generally not necessary for shoot induction (VAN AARTRIJK & VAN DER LINDE, 1986).

Most monocotyledons require auxins and cytokinins, in combination, for maximum shoot production (HUSSEY, 1978). In *Narcissus* cultivars, shoot production was stimulated by including both auxin and cytokinin in the medium (SEABROOK *et al.*, 1976; BERGOÑÓN *et al.*, 1992), although higher than normal levels were required to effect a response (HUSSEY, 1982[a] and 1982[b]). The stimulation of direct organogenesis by NAA, BA and kinetin was also reported for *Leucojum aestivum* (STANILOVA *et al.*, 1994).

Thus, for most micropropagation protocols involving bulbous plants, shoot induction is stimulated by auxin and / or cytokinin. Various plant growth regulators are used since different plants and explant sources thereof, show different sensitivities to the hormones. Generally NAA is better than 2,4-D in the culture of monocotyledons (HUGHES, 1981), although, in the case of *Narcissus* (HUGHES, 1981), 2,4-D was found to be more effective than NAA for shoot induction. Plantlets form adventitiously from one or a few less specialized cells in the explant (VAN AARTRIJK & VAN DER LINDE, 1986).

MATERIALS AND METHODS

Explants from the inflorescences, leaves and bulbs of *C. moorei* were inoculated onto a Murashige and Skoog (1962) (M&S) basal medium containing various combinations of auxin and cytokinin. NAA and 2,4-D were used as sources of auxin and BA as the cytokinin, in preparing the following hormone trials.

Anthers
NAA:BA, mg ℓ^{-1}(1:2)

Filaments
NAA:BA, mg ℓ^{-1}(0:0, 0:1, 0:5, 1:0, 1:1, 1:2, 1:5, 5:0, 5:1, 5:5)

Petals
NAA:BA, mg ℓ^{-1}(0:0, 0:1, 0:5, 1:0, 1:1, 1:2, 1:5, 5:0, 5:1, 5:5)

Base of the perianth tube
The medium was not supplemented with hormones.

Ovaries
NAA:BA, mg ℓ^{-1} (0:0, 0:0.1, 0:1, 0:5, 0.1:0, 0.1:0.1, 0.1:1, 0.1:5, 1:0, 1:0.1, 1:1, 1:5, 5:0, 5:0.1, 5:1, 5:5)

Pedicels
NAA:BA, mg ℓ^{-1} (0:0, 0:0.1, 0:1, 0:5, 0.1:0, 0.1:0.1, 0.1:1, 0.1:5, 1:0, 1:0.1, 1:1, 1:5, 5:0, 5:0.1, 5:1, 5:5)

Pedicel-Peduncles
2,4-D:BA, mg ℓ^{-1} (0:0, 0:1, 0:2, 1:0, 1:1, 1:2, 2:0, 2:1, 2:2)

Peduncles
NAA:BA, mg ℓ^{-1} (0:0, 0:1, 0:5, 0.1:0, 0.1:1, 0.1:5, 1:0, 1:1, 1:5)
2,4-D:BA, mg ℓ^{-1} (0:0, 0:1, 0:2, 1:0, 1:1, 1:2, 2:0, 2:1, 2:2)

Leaves
NAA:BA, mg ℓ^{-1} (0.5:1)

Bulb scales
NAA:BA, mg ℓ^{-1} (0:0, 0:0.1, 0:1, 0.1:0, 0.1:0.1, 0.1:1, 1:0, 1:0.1, 1:1)

Twin-scales
NAA:BA, mg ℓ^{-1} (0:0, 0:0.1, 0:1, 0.1:0, 0.1:0.1, 0.1:1, 1:0, 1:0.1, 1:1)
NAA:BA mg ℓ^{-1} (0:0, 0:0.5, 0:1, 0:2, 0.5:0, 0.5:1, 0.5:2, 1:0, 1:1, 1:2)

RESULTS

Anthers
No growth response was obtained for the anthers.

Filaments
There was no differential response of the filaments to the hormone treatments. They all turned brown without producing new growth.

Petals
No growth of the petals was observed after supplementing the basal medium with plant growth regulators. The petals discoloured easily with the release of anthocyanins into the medium. Better tissue preservation was noted when the highest levels of hormones were used.

Base of the perianth tube
Initially these explants produced a wound callus at the cut surface. No further growth occurred as the callus turned brown.

Ovaries
The ovaries became swollen and formed protuberances around the perimeter. The epidermis sometimes split and curled back exposing the ovary wall. This tissue later turned brown. Translucent to white wound callus appeared around the cut epidermis and as a "coating" on the upper cut surface. The callus was crystalline and not nodulated. It became dry and brown with age. Finger-like projections of callus also extended into the medium at the base of the ovary (Figure 3.4). No shoots developed in any of the treatments.

Pedicels

The pedicels, like the ovary explants, became swollen in culture and produced wound callus on the cut surfaces. The wound callus did not proliferate but turned brown. Sometimes the callus extended into the medium as finger-like projections. Nodular callus developed on explants grown on media supplemented with 1 mg ℓ^{-1} BA and 5 mg ℓ^{-1} NAA as well as 5 mg ℓ^{-1} BA and 5 mg ℓ^{-1} NAA (Figure 3.4). This was either cream or brown in colour. Both lines of callus were maintained on the induction medium.

Pedicel-Peduncles

Callus formed in only one treatment; that containing 1 mg ℓ^{-1} 2,4-D and no BA. The callus was cream, compact and nodular and developed in all replicates. In other treatments, the explants responded by producing a wound callus on the upper cut surface. Although this was initially white, it later turned brown with age. Some explants produced finger-like callus which projected into the medium.

Peduncles

Peduncle explants did not respond to a range of hormone concentrations and combinations. Only the cytokinin, BA, was effective in inducing callus and shoot production and this at the highest concentration used, namely 5 mg ℓ^{-1} BA. The response was, however, poor, with one explant producing callus and another shoots. Unlike the typical wounding response in most other treatments, the callus that developed on the upper cut surface of peduncles in 5 mg ℓ^{-1} BA, was nodular (Figure 3.4). The shoots were translucent to green and occurred around the epidermal surface. In other hormone trials using 2,4-D as the source of auxin rather than NAA, callus developed in two treatments containing 1 mg ℓ^{-1} BA and 2 mg ℓ^{-1} BA respectively. The callus was either crystalline and green (1 mg ℓ^{-1} BA) or translucent and granular (2 mg ℓ^{-1} BA). All other explants initially produced a wound callus. Sometimes this formed finger-like projections or basal nodules in the medium or grew as patches of white crystalline callus on the surface of the medium. The wound callus did not proliferate further but turned brown.

Leaves

Leaf explants produced a crystalline callus at the midrib and vein endings in response to wounding (Figure 3.4).

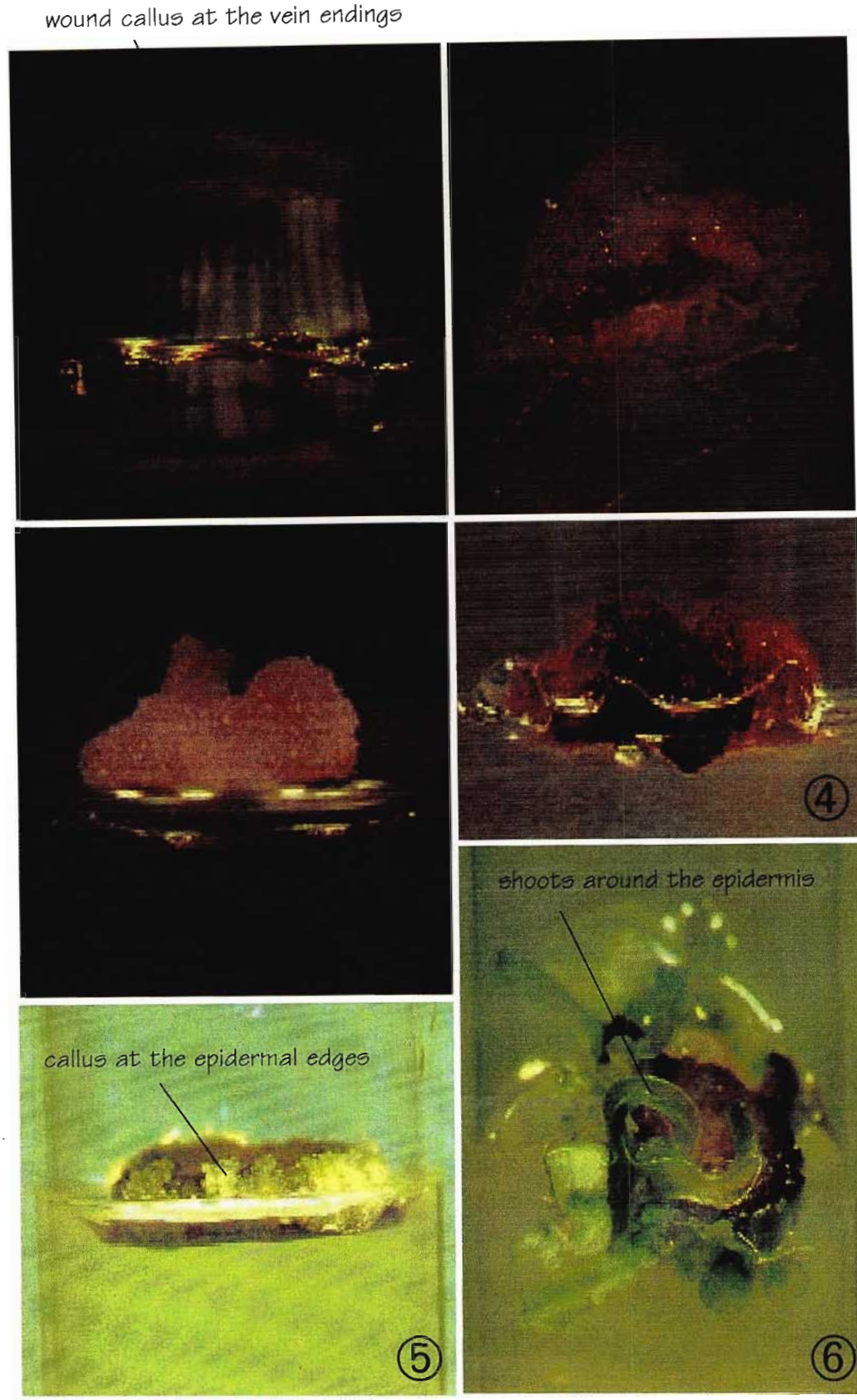


Figure 3.4: Growth response of leaf and inflorescence explants on hormone-supplemented media

1. Crystalline wound callus on leaf explants
2. Wound callus on sections of ovary tissue
3. Nodular callus (cream) on pedicels
4. Nodular callus (brown) on pedicels
5. Nodular callus on peduncles
6. Shoots on peduncle

Bulb scales

Bulb scales, in all hormone treatments, turned black and showed no growth response. However, where the medium was supplemented with 1 mg ℓ^{-1} BA and 1 mg ℓ^{-1} NAA, the bulb scales turned dark green and became nodular with short hairy roots. Some scales produced a green crystalline wound callus.

Twin-scales

Plantlet regeneration occurred in the absence of plant growth regulators but also in treatments containing low concentrations of BA and NAA (Figure 3.5). Shoots formed in the axes of the twin-scales, near the basal plate. NAA, at a concentration of 0.5 or 1 mg ℓ^{-1}, either alone or in combination with BA, induced abnormal organogenesis, rather than plantlet production. The green scales became swollen, translucent and produced either crystalline callus or short hairy roots over their surfaces (Figure 3.5). An increase in the level of BA in the medium resulted in better shoot production in terms of both the number of explants responding and the mean number of shoots per explant (Figure 3.6).

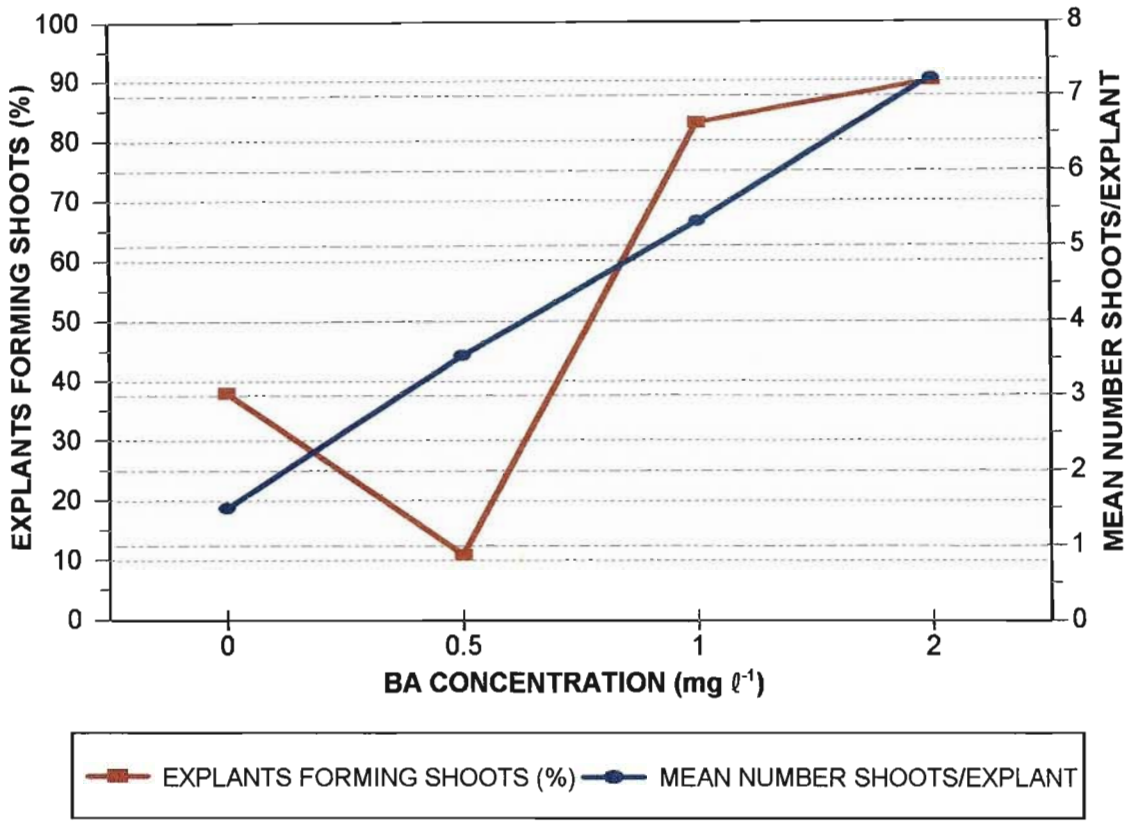
The growth of inflorescence- and bulb-derived explants in response to hormonal supplements is summarized below (Table 3.6).

Table 3.6: Growth of selected explants on hormone-supplemented media

Explant	Growth response	Hormones
Anthers	x	-
Filaments	x	-
Petals	x	-
Base of perianth tube	x	-
Ovary	x	-
Pedicel	Callus	BA (1 mg ℓ^{-1}) and NAA (5 mg ℓ^{-1}) BA (5 mg ℓ^{-1}) and NAA (5 mg ℓ^{-1})
Pedicel-Peduncle	Callus	2,4-D (1 mg ℓ^{-1})
Peduncle	Callus Shoots	BA (1, 2 and 5 mg ℓ^{-1}) BA (5 mg ℓ^{-1})
Leaves	x	-
Bulb scales	x	-
Twin-scales	Shoots	No hormones required OR BA (0.5, 1 or 2 mg ℓ^{-1})

Figure 3.5: Growth response of twin-scales on hormone-supplemented media

1. Shoots in the axis of adjacent bulb scales; arising from the basal plate on a hormone-free medium
2. Abnormal organogenesis induced by NAA alone or in combination with BA



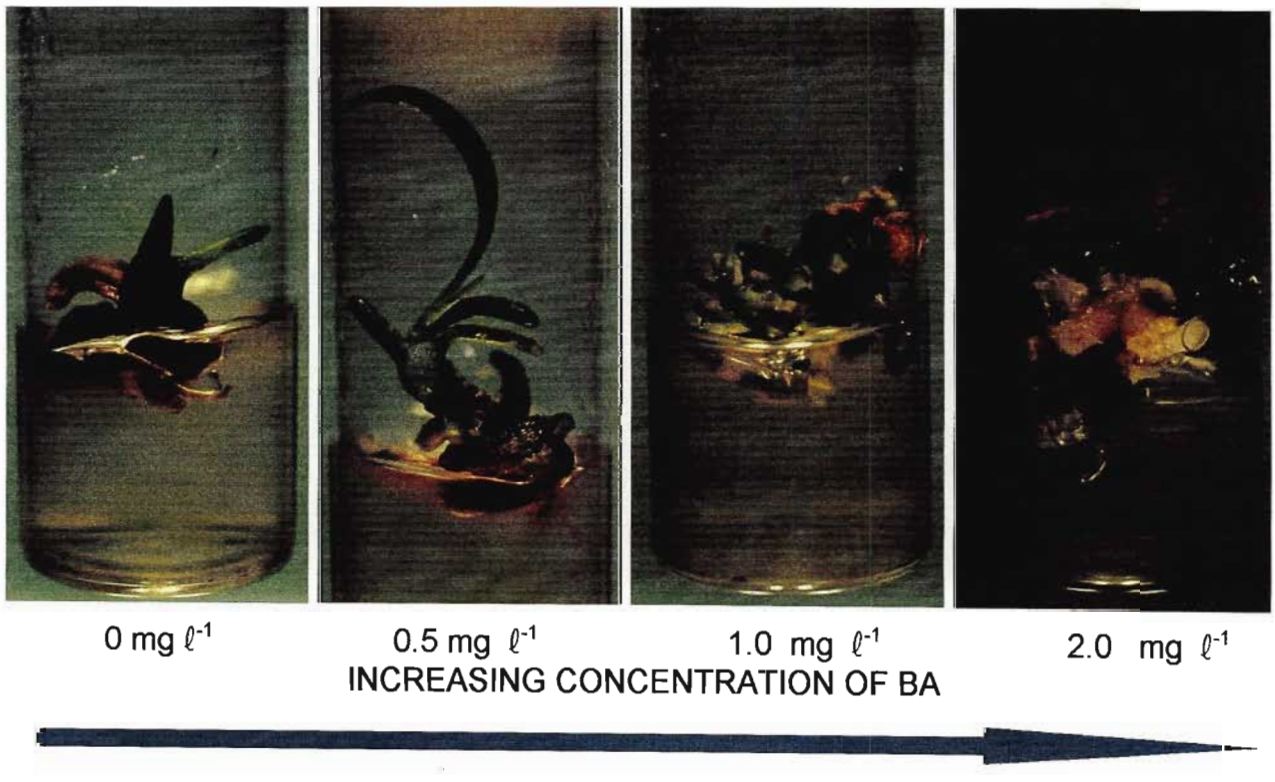


Figure 3.6: The effect of increasing the BA concentration on shoot production from twin-scales

DISCUSSION

The auxin, NAA, and cytokinin, BA, failed to stimulate callus production and / or organogenesis from the anthers, filaments, petals, perianth tube, ovaries, leaves and bulb scales, in the concentration ranges tested. Most explants responded to wounding by producing a crystalline callus on their cut surfaces. This is typical of many plant tissues when they are excised during explantation, especially when the ratio of cut surface to volume is high. The act of injuring plant cells by cutting, produces a stimulus to cell division, which results in the disorganized growth of plant cells called callus (GEORGE, 1993). Callus varies in its compaction and degree of morphogenetic potential; due largely to the origin and age of the explant tissue. Friable callus is loosely packed and generally non-morphogenic (GEORGE, 1993), as was the case for the explants described above. In tissue culture studies involving *Clivia miniata*, a crystalline callus was also observed on explants from several sources and was shown to have no further morphogenetic response (FINNIE, 1988).

Although differentiated cells of somatic tissues are, in theory, capable of regenerating a complete new plant, explants that are derived from determinate or non-meristematic tissues generally have a low regenerative capacity, even when supplied with exogenous hormones. In an investigation of the totipotency of tissue explants among members of the Liliaceae, Iridaceae and Amaryllidaceae, Hussey (1975) concluded that while plantlets could be induced on the leaves of liliaceous species, this was not possible for the amaryllidaceous species selected. A similar trend was noted for plantlet induction from ovary wall tissue. In other studies, leaf tissue of several *Cyrtanthus* species also did not yield positive results by producing plants (McALISTER *et al.*, 1998[b]) nor did leaf, anther, filament and petal tissue of *Hippeastrum hybridum* (FOUNTAIN & O'ROURKE, 1980). Single scales from the bulbs of amaryllidaceous species are, in general, not productive. For example, Mullin (1970) used bulb scale tissue of a *Crinum* sp. and observed limited growth after two months on BM-CM (basal medium with coconut milk) media containing 6 mg ℓ^{-1} 2,4-D. The scales were unsuccessfully transferred to 0.1 or 1 mg ℓ^{-1} NAA as blackening of the older tissue was noted. Several authors maintain that the lack of a regenerative response from bulb scale tissue is because meristematic activity is restricted to the basal plate - found only in twin-scale explants (PIERIK & IPPEL, 1977; CHOW *et al.*, 1993). Yet, there are other reports where leaves (STEINITZ & YAHEL, 1982; LIN *et al.*, 1997; LIN *et al.*, 1998), leaf bases, ovaries and disks (basal plate) produce adventitious shoots when supplemented with auxin and cytokinin (HUSSEY, 1975, 1976, 1980 and 1982; SEABROOK *et al.*, 1976; SEABROOK & CUMMING, 1977 and 1982; HOSOKI & ASAHIRA, 1980). In those instances where leaves are used, explants are mostly derived from the leaf base which is in close contact with the meristematic basal plate (SEABROOK *et al.*, 1976; SEABROOK & CUMMING, 1977 and 1982; HUSSEY, 1980 and 1982). The use of floral material in regenerating plantlets has met with success particularly

in the Liliaceae. Arzatefernandez *et al.* (1997), for example, propagated *Lilium longiflorum* using filaments with attached anthers. Indirect adventitious organogenesis has also been described for *Crinum* sp. Buds, which resembled bulbils, and plantlets were initiated from callus on ovary explants (YAMADA, 1963; cited by KRIKORIAN & KANN, 1986).

Callus developed on the pedicel explants, at the junction of the pedicel and peduncle and on the cut surface of only one peduncle explant grown on hormone-supplemented media. The induction of callus varied according to the type and concentration of hormone used. The highest concentration of auxin tested, i.e. 5 mg ℓ^{-1} NAA, in combination with BA, stimulated callus production on the pedicels. As an alternative source of auxin, 2,4-D successfully induced the production of callus from the junction of pedicel-peduncle explants. Callus development on the peduncles was, however, initiated in response to a range of cytokinin concentrations; 1, 2 and 5 mg ℓ^{-1} BA. "Production and maintenance of vigorous callus from monocotyledons have traditionally been viewed as difficult (KRIKORIAN & KATZ, 1968), and this view is in large measure still justified (HUNAULT, 1978a, b. 1979a, b)" (KRIKORIAN & KANN, 1986). Even within the monocotyledons, there is a distinct difference in the capacity of members of the Liliaceae, Iridaceae and Amaryllidaceae to form callus; this being most difficult for the Amaryllidaceae (HUSSEY, 1975). *Narcissus* tissues, for example, do not readily form callus (SEABROOK *et al.*, 1976) and only with difficulty from the ovary wall (HUSSEY, 1975). Thus *Narcissus* cultivars may require high levels of auxin to induce callus formation (HUSSEY, 1975; SEABROOK *et al.*, 1976). Other explants form callus more readily, notably the flower stalks of *Narcissus* cultivars (HOSOKI & ASAHIRA, 1980). Differences in the regeneration potential of tissues belonging to the inflorescence appear to be quite common and may be related to the orientation or polarity of the explants and thus to differences in the levels of endogenous hormones (see WRIGHT, 1981; cited by ALDERSON & RICE, 1986). Auxin promotes the efflux of H^+ in basal cells and so maintains an electrical potential difference between the shoot apex (positive) and base (negative). This, in turn, is thought to maintain and reinforce the transport of auxin in a polar direction (RAVEN, 1979; cited by GEORGE, 1993). The effects of polarity have been reported for *Gladiolus* inflorescence stalks. Segments taken from the basal ends produced callus and roots but at the distal end formed buds and cormlets (ZIV *et al.,* 1970). Fountain and O'Rourke (1980), on the other hand, found that 'explant productivity' increased towards the proximinal end of the flower stem and that the pedicel tissue was more productive than the ovary tissue in producing callus and plantlets. Polarity effects have even been recorded within the pedicel. Explants excised nearest the receptacle of *Lilium longiflorum* produced five times as many buds than those towards the proximal end (LIU & BURGER, 1986; cited by GEORGE, 1993). The proximity of the explant to naturally occurring meristems may also affect its regenerative response. Although both auxins and cytokinins are necessary in regulating the cell cycle, the onset of mitosis is triggered only when cytokinins are present (GEORGE, 1993). This implies

that there are cytokinins present in floral meristems in sufficient quantities to induce growth and that exogenous applications would be growth-inhibiting. This perhaps explains why explants from the pedicel-peduncle junction of *Crinum moorei* did not require additional cytokinin to stimulate callus production.

Although callus forms naturally in many explants in reaction to wounding, the addition of supplementary auxin to the culture medium is necessary to sustain cell division (HUSSEY, 1978; HOSOKI & ASAHIRA, 1980). Even when supplied, callus growth is sometimes poor. This was noted in the present study and has also been reported for *Nerine bowdenii* (JACOBS *et al.*, 1992). Thus higher than normal levels of auxins may be required for callus production in monocotyledons, e.g. 2,4-D is used in the range of 2.0 - 10.0 mg ℓ^{-1} (GEORGE, 1993). Sellés *et al.* (1997) found that low concentrations of auxin could successfully induce callus on *Narcissus confusus* seeds, whereas it was previously reported that high concentrations (10 mg ℓ^{-1} 2,4-D) were necessary. For some species, like *C. moorei*, a cytokinin is needed to initiate growth. The pedicel-peduncle explants of *Nerine* also required 2,4-D and BA in combination to induce callus production (LILIEN-KIPNIS *et al.*, 1992; ZIV *et al.*, 1995). It appears that this requirement is not absolute where pedicel-peduncle explants are concerned, for 2,4-D rather than BA, could induce callus from the junction of the pedicel and peduncle of *C. moorei.*

The potential of callus for use in adventitious plant production is limited by the fact that few ornamental plants produce totipotent calli i.e. they do not produce shoots, and when this does occur, their capacity for regeneration decreases with age (as totipotent cells are diluted out with repeated subculture) while genetic abnormalities increase. For example, although callus was initiated from bulb scales of *Crinum asiaticum*, it did not show an organogenic response (MUJIB *et al.*, 1996). Despite this fact, there were early indications that plantlets could be produced from callus-derived buds in monocotyledons (BEAL, 1937; cited by KRIKORIAN & KANN, 1986). These were obtained, for example, from the "callus-like" tissues on the leaves of *Haemanthus* (HALL & TOMLINSON, 1973). In several species of lilies, irises and amaryllids, plantlets could also be induced from callus (HUSSEY, 1976[b]). Plant regeneration from filament and anther-derived callus was found to be highly efficient in *Lilium longiflorum* and thus suitable for mass propagation (ARZATEFERNANDEZ *et al.*, 1997). Pierik and Steegmans (1986) report that callus formation always preceded bulblet regeneration from floral stems of *Nerine bowdenii* while callus from floral tissues of *Crinum*, especially ovaries, was shown to be capable of producing buds and plantlets (YAMADA, 1963; cited by KRIKORIAN & KANN, 1986). Mature seeds of *Narcissus confusus* yielded callus which was later shown to be totipotent. For when cytokinins were added, shoot-buds regenerated on the surface of the callus (SELLÉS *et al.*, 1997).

Despite its limits of applicability, callus production may prove useful in cases where there is little explant material (HUSSEY, 1978) and in the induction of somatic embryos. These have potential in automated systems involving liquid cultures and bioreactors. Already, success has been achieved for *Nerine* sp. where callus was used for the induction of somatic embryos by removing PAC and adding 2iP (LILIEN-KIPNIS *et al.*, 1992). In the case of *Crinum asiaticum*, compact flower bud calluses yielded somatic embryos when supplied with BA and 2,4-D and retained their embryogenic potential for more than a year (MUJIB *et al.*, 1996). Sellés *et al.* (1997) also found that callus had a greater capacity to form shoots and that it retained its regenerative ability for more than 20 months. Since the aim of the work was to produce galanthamine - a secondary metabolite - *in vitro*, the potential for genetic abnormalities through indirect organogenesis may not have been so much of a problem.

The most responsive tissues are those obtained from the peduncle and from the bulbs of *Crinum moorei*, in the form of twin-scales. Both explants produced shoots. This is consistent with reports for several other amaryllidaceous species (HUSSEY, 1975; VAN AARTRIJK & VAN DER LINDE, 1986; ZIV & LILIEN-KIPNIS, 2000). The scapes and peduncles, for example, were the most productive tissues for the propagation of *Hippeastrum* hybrids (SEABROOK & CUMMING, 1977). On the other hand, no plantlets could be obtained from tissues other than the basal meristem in twin-scale segments of *Narcissus* and *Hippeastrum* (HUSSEY, 1975). The twin-scales were also the most consistently reactive explants for *Nerine* (HUSSEY, 1980). Thus, for most bulbous plants, micropropagation strategies entail enhancing shoot formation from axillary buds (KRIKORIAN & KANN, 1986), that is, direct organogenesis, without concomitant callus production.

Adventitious shoots are known to be produced from floral stem explants in species like tulip (RICE *et al.*, 1983). Those from peduncle explants of *C. moorei*, were produced at relatively high cytokinin levels and in the absence of auxin. Increased adventitious bud formation has also been reported following cytokinin treatment of flower stalks of *Narcissus* cultivars (HUSSEY, 1976) and *Nerine bowdenii* (JACOBS *et al.* 1992). The stimulatory effect of cytokinins on the number of plants produced has been described in a number of other reports (VAN AARTRIJK & VAN DER LINDE, 1986). In other monocotyledonous bulbs and corms, young flower stems respond to auxin or auxin and cytokinin (HUSSEY, 1982). Low concentrations of auxin ($< 10^{-6}$ M) increase the induction of cell division and plantlets (VAN AARTRIJK & VAN DER LINDE, 1986). Occasionally, plantlets formed on floral stem segments of *Hippeastrum* in response to medium concentrations of NAA (HUSSEY, 1980); the auxins IAA and 2,4-D were unsuccessful. *Ipheion uniflorum* also has a requirement for auxin (HUSSEY, 1975). Other studies showed that NAA was essential for shoot regeneration from floral stem tissues of *Hippeastrum* and *Nerine*, and that BA was not as important (ALDERSON & RICE, 1986). However, Seabrook and Cumming (1977), report that peduncles

of *Hippeastrum* spp. hybrids were the most responsive tissues in culture, forming plantlets when 1 mg ℓ^{-1} 2, 4-D and 1 mg ℓ^{-1} BA were added to the culture medium. A combination of equal concentrations of 2,4-D and BA was used to regenerate buds on *Nerine sarniensis* explants (ZIV & LILIEN-KIPNIS, 2000). Shoot regeneration from tulip floral stem explants is also readily achieved on media containing 1 mg ℓ^{-1} of both NAA and BA (ALDERSON & RICE, 1986). Regeneration of bulblets from floral stems of *Nerine bowdenii* depends on cytokinin in addition to auxin i.e. 1 mg ℓ^{-1} BA and 0.5 mg ℓ^{-1} NAA (PIERIK & STEEGMANS, 1986). A higher cytokinin to auxin ratio was required for the induction of adventitious shoots from the scapes of *Narcissus* cultivars (SEABROOK *et al.*, 1976; HUSSEY, 1977; HOSOKI & ASAHIRA, 1980; HUSSEY, 1980; HUSSEY, 1982[b]; HYANGYOUNG *et al.*, 1997), *Nerine* spp. (HUSSEY, 1980) and *Gladiolus*; from the pedicel-peduncle junction of *Hyacinthus* and *Eucrosia* (ZIV & LILIEN-KIPNIS, 2000); and from the immature scapes of *Amaryllis belladonna* (DE BRUYN *et al.*, 1992). As many as 10 buds formed on inflorescence explants when the kinetin to NAA ratio was high (ZIV & LILIEN-KIPNIS, 2000). Various hormone combinations were used successfully in regenerating adventitious plantlets from floral stems of *Crinum macowanii*. The highest number of shoots were regenerated in media containing kinetin (4.65 µM) and either IAA (0.57 µM) or NAA (0.45 µM), or a combination of BA (4.44 µM) and 2,4-D (0.45 µM) (SLABBERT *et al.*, 1995). The discrepancies that exist in the literature regarding the type and concentrations of hormones used to induce organogenesis from inflorescence parts can, perhaps, be explained on the basis of tissue-dependent factors. Ziv and Lilien-Kipnis (2000) found that the number of regenerants not only varied with the genus but depended on the developmental stage of the inflorescence at the time at which it was excised. The addition of auxin and cytokinin may, thus, reinforce existing polarity effects, induce growth from unresponsive tissues or reverse polar trends (GEORGE, 1993).

Twin-scales spontaneously produce shoots from pre-existing axillary meristems (GROOTAARTS *et al.*, 1981) in the absence of plant growth regulators (STEINITZ & YAHEL, 1982; ALDERSON & RICE, 1986; BERGOÑÓN *et al.*, 1996). In fact, natural mechanisms of axillary or adventitious bud formation are exploited in the micropropagation of bulbous ornamentals (HUSSEY, 1976) because they readily form shoots. As this occurs from large multicellular meristems involving several layers of tissue at the base of scales, plantlets are seldom affected by somaclonal changes (YEOMAN, 1986). While cytokinins may be needed to induce adventitious plantlet formation from peduncles (and other inflorescence parts), these compounds are not essential when explants from the bulbs are used (see also VAN AARTRIJK & VAN DER LINDE, 1986). For example, plant growth regulators were not required for shoot induction from twin-scales of *C. moorei* or *C. macowanii*, where the average number of plantlets regenerated without plant growth regulators compared favourably with the best hormone treatments (SLABBERT *et al.*, 1993). At high concentrations a combination of auxin and cytokinin, in fact, reduced shoot formation in *C. moorei*. This was

accompanied by abnormal organogenesis. The addition of plant growth regulators to the medium was also found to reduce the number of shoots per explant in *Iris hollandica* (VAN DER LINDE *et al.*, 1988). The percentage of explants which developed callus was also higher. Other studies have shown that an increase in the concentration of BA leads to a decrease in shoot production (McALISTER *et al.*, 1998[b]) or plantlets which are reduced in size (CUSTERS & BERGERVOET, 1992) and that BA and NAA, in interaction, adversely affect the numbers of shoots produced (McALISTER *et al.*, 1998[a]). In the case of *Hippeastrum hybridum*, infrequent bud development was noted after using NAA at high concentrations (MII *et al.*, 1974). The addition of NAA (at 0 and 5 mg ℓ^{-1} BA) also inhibited plantlet formation from twin-scales of *Crinum macowanii* (SLABBERT *et al.*, 1993) as it did to shoot induction from tri-scales of *Crinum* 'Ellen Bosanquet' (ULRICH *et al.*, 1999). Its presence also has a detrimental effect on the formation of shoots and roots of *Babiana* spp. seedlings (McALISTER *et al.*, 1998[a]). In other flower-bulb crops, ABA was found to inhibit plantlet induction (VAN AARTRIJK & VAN DER LINDE, 1986). High concentrations of auxin may also lead to abnormal shoot (ULRICH *et al.*, 1999) and root development (MII *et al.*, 1974). Differences in the requirements for plant growth regulators may be attributed to apical dominance, which inhibits the development of axillary meristems to varying degrees but which, in culture, can be released using different combinations of hormones. This depends on the type of plant and how much it naturally branches (YEOMAN, 1986). Endogenous hormones are often present in sufficient quantities, making the addition of growth regulators unnecessary (HUSSEY, 1982). It is generally accepted, however, that the inclusion of cytokinins in the medium promotes the outgrowth of axillaries in cultured buds (YEOMAN, 1986). For example, IBA alone promoted growth from twin-scales of *Eucharis grandiflora* (PIERIK *et al.*, 1983) and *Nerine bowdenii* (PIERIK & IPPEL, 1977). In *Gladiolus* species BA stimulated shoot induction from corms but was strongly influenced by sucrose and temperature (DE BRUYN & FERREIRA, 1992). Compared to the Iridaceae, amaryllidaceous species may require higher cytokinin levels to promote branching (HUSSEY, 1976). This was reported for *Nerine bowdenii* (JACOBS *et al.*, 1992). In the case of *C. moorei*, cytokinins could be used to improve shoot initiation when used alone and at levels of 1 and 2 mg ℓ^{-1}. Much higher levels of BA (22.2 µM and 35.5 µM) were necessary for increased shoot production in *Crinum* 'Ellen Bosanquet' (ULRICH *et al.*, 1999). In other instances cytokinins, together with auxins, were found to be stimulatory (SEABROOK & CUMMING, 1977; HUSSEY, 1980; SQUIRES & LANGTON, 1990; BERGOÑÓN *et al.*, 1992; CUSTERS & BERGERVOET, 1992; DE BRUYN *et al.*, 1992; HOL & VAN DER LINDE, 1992; STANILOVA *et al.*, 1994; McALISTER *et al.*, 1998[a]). Slabbert *et al.* (1993) report that plantlets could be initiated from twin-scales of *Crinum macowanii* using a range of BA and NAA concentrations but that increasing the concentration of BA from 0 - 20 mg ℓ^{-1} (88.8 µM) resulted in a higher average number of shoots per explant. High cytokinin to auxin ratios also proved effective in inducing shoots in

other bulbous species (MII *et al.*, 1974; SEABROOK *et al.*, 1976; HUSSEY, 1982; TAYLOR & VAN STADEN, 1997).

CONCLUSIONS

Peduncles and twin-scales from *C. moorei* bulbs are the best sites for explantation of regenerative materials. As has been shown in other amaryllidaceous species, totipotency is not restricted to the tissues near the basal plate. But, unlike the twin-scales, which readily produce shoots, peduncle explants require additional cytokinins to initiate cell division. At concentrations of 5 mg ℓ^{-1} BA, calli or adventitious shoots develop from the peduncles. Although callus is "less attractive in view of the greater risks of incurring abnormal propagules" (HUSSEY, 1982[a]), the fact that peduncles produce multiple shoots, is perhaps worthy of consideration in the selection of a suitable explant. Peduncles and twin-scales were, therefore, subjected to further investigation to determine the effect of several factors on growth and morphogenesis.

Chapter 4
THE PRODUCTION OF PROPAGULES

INTRODUCTION

Stage II of the micropropagation scheme involves producing an increased number of propagules. For geophytes, this is achieved through shoot clump cultures, e.g. *Narcissus* sp. (CHOW *et al.*, 1992[b]; BERGOÑÓN *et al.*, 1996; SELLÉS *et al.*, 1997) or by inducing the formation of storage organs and using these to initiate further cultures (GEORGE, 1993). Rarely is callus used, although there are instances where it readily forms in response to wounding and endogenous or exogenous growth factors and where it can be manipulated to form adventitious shoots. It may be preferred to other propagules since it is easy to manipulate at different developmental stages (ZIV *et al.*, 1995) and can be scaled-up for mechanization (ZIV *et al.*, 1995). Direct organogenesis is, however, preferred to callus induction (PECK & CUMMING, 1986) for several reasons. Firstly, bulblets regenerate more quickly from bulb scales than from callus (SELLÉS *et al.*, 1997) because they have a stronger regenerative capacity. This effectively reduces the number of subculture steps. Compared to bulblet regeneration from callus, which took 10 - 12 months, that from twin-scales of *Narcissus confusus* was achieved in six months (SELLÉS *et al.*, 1997). Secondly, the immediate development of bulblets ensures that the genetic uniformity of the propagules is not affected (PECK & CUMMING, 1986). Techniques that exploit the natural mechanisms of axillary and adventitious shoot formation are useful for long term multiplication and storage of selected genotypes since they combine the maximum of genetic stability with the minimum loss of totipotency (HUSSEY, 1976[a]). This cannot be said of callus or suspension cultures which accumulate chromosomal abnormalities over time and lose their potential to regenerate plants. Indirect organogenesis is thus unsuitable for clonal propagation. Thirdly, the induction of storage organs *in vitro* results in improved plantlet survival upon transfer to the soil (PECK & CUMMING, 1986) and may significantly shorten the period until flowering - which takes several seasons when bulbs are micropropagated by traditional means (VISHNEVETSKY *et al.*, 1997).

Shoots and bulblets arise from pre-existing axillary meristems and adventitious shoots (GROOTAARTS *et al.*, 1981) in the axes of twin-scales e.g. *Nerine bowdenii* (GROOTAARTS *et al.*, 1981) where the scales are implanted on the basal plate e.g. *Eucharis grandiflora* (PIERIK *et al.*, 1983). Chow *et al.* (1993) suggest that the basal plate is absolutely necessary

for regeneration and only when twin-scales are used are high multiplication rates achieved (LANGENS-GERRITS & NASHIMOTO, 1997). This is due to the greater density of meristematic cells in the basal plate compared to the apical parts of the scales (STANILOVA *et al.*, 1994). Shoots frequently develop on the abaxial surface of the inner scale e.g. *Hippeastrum hybridum* (OKUBO *et al.*, 1990) from at least two superficial layers of meristematic cells near the basal plate e.g. *Narcissus* (HUSSEY, 1982[b]). Here, meristematic cells are characterised by the absence of starch grains (GROOTAARTS *et al.*, 1981). De Bruyn *et al.* (1992) noted that the protuberances arose from both the epidermal and subepidermal tissue on the abaxial side of the inner scale. Yanagawa and Sakanishi (1990; cited by HUANG *et al.*, 1990) recognised the importance of the proximity of the epidermal and subepidermal cells to the vascular bundles since shooting was frequently observed from epidermal tissue nearest the vascular bundles in *Hymenocallis*. Although new shoots develop on the abaxial side of the inner scale, vascular connections are made with outer scale (OKUBO *et al.*, 1990).

Shoot initiation in shoot clump cultures occurs within the amorphous achlorophyllous tissue at the base of the clump. Apparently this has a structure similar to the basal plate with a complex vascularization and small, densely packed parenchyma cells. It is therefore thought to be genetically uniform (CHOW *et al.*, 1993).

Shoots form adventitiously on immature floral stem disks (PIERIK & STEEGMANS, 1986) in species like tulip (RICE *et al.*, 1983). They arise from the epidermal layers and some adjacent cells of the parenchymatous ground tissue as in *Hippeastrum* (SMITS *et al.*, 1989) and *Gladiolus* (ZIV & LILIEN-KIPNIS, 2000). Later they develop a vegetative centre or bulb primordium (RICE *et al.*, 1983). The multicellular origin of bulbs from floral stems is important in establishing clones, since there is little chance that the bulblets differ genetically from the parent tissue (SMITS *et al.*, 1989).

Callus which develops from the peduncle e.g. *Nerine bowdenii* (JACOBS *et al.*, 1992) or from pedicel-peduncle explants e.g. *Nerine* (LILIEN-KIPNIS *et al.*, 1992) is in some instances organogenic. In the examples cited, callus either formed bulblets (JACOBS *et al.*, 1992) or, through induction, formed meristematic clusters. The proembryogenic masses produced somatic embryos which were later successfully germinated (LILIEN-KIPNIS *et al.*, 1992 and 1994).

The initiation of growth during stage one of this study resulted in callus and shoot formation from peduncle explants as well as shoots in the axes of twin-scales. Growth and morphogenesis of these propagules were investigated in relation to both tissue-dependent factors and the culture environment.

Regeneration from floral stem explants: Factors affecting growth and morphogenesis

INTRODUCTION

Direct organogenesis and callus production were achieved using the peduncles of *C. moorei.* Where this has been reported for other genera in the Amaryllidaceae, several factors have been identified as affecting growth and morphogenesis, namely: explant age, position, size and polarity; plant growth regulators (as discussed in the previous chapter); sucrose; light and temperature (PIERIK *et al.*, 1990).

EXPLANT AGE

Introduction

The regenerative potential of explants from the inflorescence stalk depends on its developmental stage at the time of excision (ZIV, 1997; ZIV & LILIEN-KIPNIS, 1997[b] and 2000). Immature floral stems (2.5 - 10 cm in length) are used more frequently than older stems since they are more successful in regenerating plantlets e.g. *Amaryllis* (DE BRUYN *et al.*, 1992) and *Nerine* (PIERIK & STEEGMANS, 1986; JACOBS *et al.*, 1992).

Materials and methods

Peduncles were harvested as they emerged from the neck of the bulb (Figure 4.1). Immature peduncles were, at first, relatively short but showed considerable extension until anthesis. Peduncles of different lengths, namely: (1) 5 - 10 cm, (2) 10 - 15 cm, (3) 15 - 20 cm and (4) 20 - 25 cm, represented the inflorescence stalk at different ages. After sterilizing in 2% NaOCℓ, the peduncle disks were placed on an M&S medium supplemented with 2 mg ℓ^{-1} BA and 0.5 mg ℓ^{-1} NAA.

Results

Only explants from the pedicel-peduncle junction of 5 cm flower stems produced shoots (Figure 4.2). The response was, however, limited to 33% of the explants. The shoots remained in the vegetative or "leafy" stage and did not undergo bulbing. No shoots or callus formed on disks taken from the peduncle alone with no variation in the response between peduncles of different ages.

Discussion

Only the youngest pedicel-peduncle explants of *C. moorei* produced shoots. Similarly, those of *Amaryllis belladonna* proved more successful than the mature tissues (DE BRUYN *et al.*, 1992). Yet, for *Nerine bowdenii*, it was not the very young stems (0.5 cm in length) but those floral stems with a length of 2.5 to 5 cm that showed the best bulbing response. Older (and longer) stems showed decreased regeneration and none at all at stem lengths of more than 20 cm (PIERIK & STEEGMANS, 1986). In general then, immature floral stems of *Nerine* are used (JACOBS *et al.*, 1992). No shoot regeneration was observed in explants from floral stems of both *Nerine* or *Hippeastrum* that had undergone extension in the light or dark (ALDERSON & RICE, 1986). It therefore appears that the regeneration response for most amaryllidaceous species, including *C. moorei*, is strongly determined by the age (length) of the original floral stem. At best, young floral stems should be used as the "determination of individual flower meristems is not (yet) canalized" (GEORGE, 1993). Organogenesis from onion flower head cultures, for example, was found to be best at the primordial stage when the capitulum consisted of ten shoots in which the oldest bud had not undergone microsporogenesis (DUNSTAN & SHORT, 1977). Although floral stems at the immature stage are sometimes dissected from non-dormant bulbs e.g. *Tulip* and *Narcissus* sp. (ZIV & LILIEN-KIPNIS, 1997[b]), this defeats the purpose of using the floral stem in order to preserve the parent bulb. The age of the mother plant may also affect the type of morphogenic response. Cauliflower curds, for example, produced callus from the floral meristems when these were harvested before the marketable stage but formed shoots at a later stage of development (CRISP & WALKEY, 1974). This is in contrast to *Allium ampeloprasum* where vegetative buds regenerated only from undifferentiated tissue between the pedicels of developing capitula (ZIV & LILIEN-KIPNIS, 1997[b]). Bulb storage is another factor that may affect the number of buds that regenerate (ZIV & LILIEN-KIPNIS, 1997[b]).

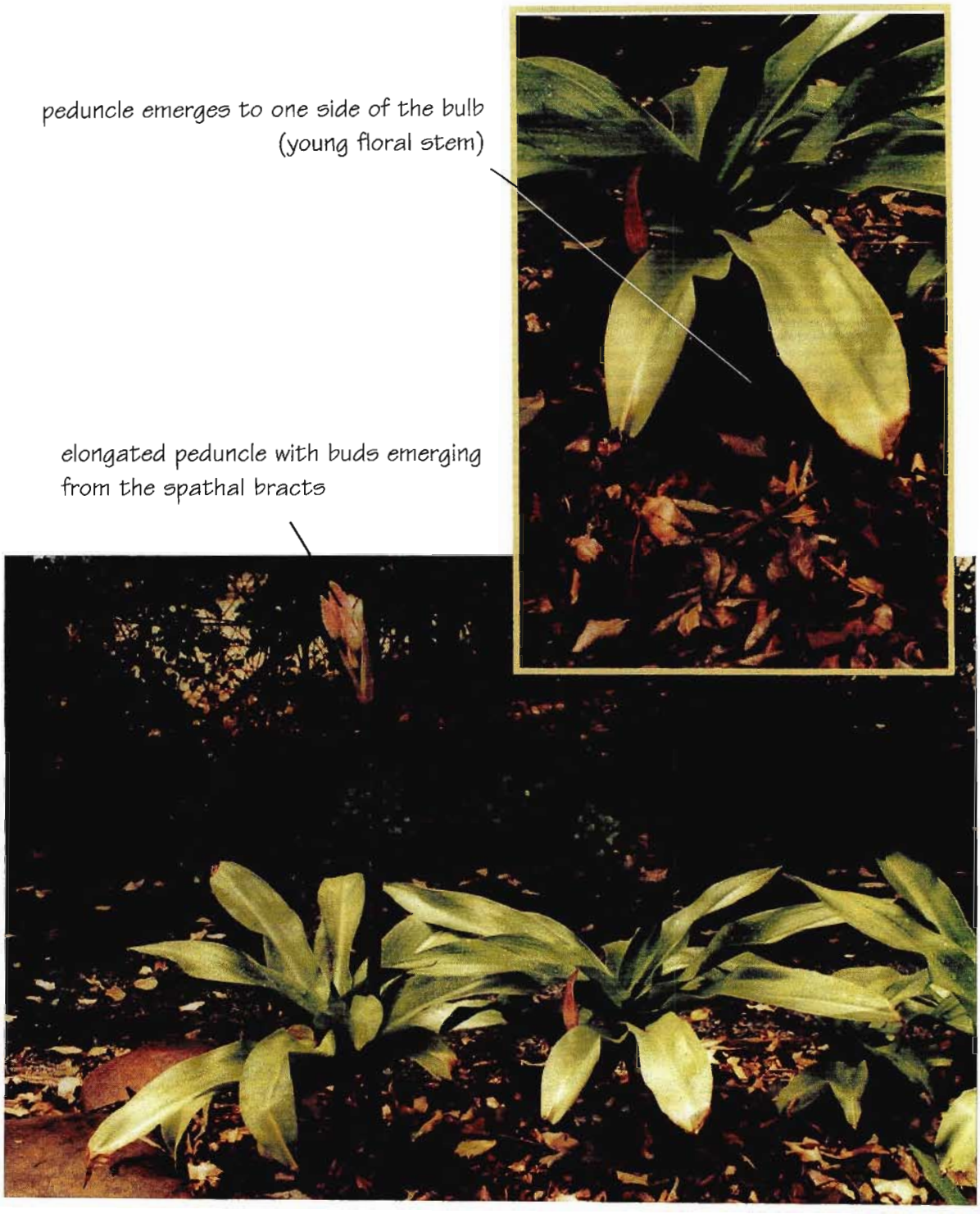


Figure 4.1: Peduncles harvested at different ages

EXPLANT POSITION

Introduction

Regeneration of bulblets is influenced by the position in the peduncle from which the explant is isolated (LE NARD & CHANTELOUBE, 1992; ZIV, 1997; ZIV & LILIEN-KIPNIS, 1997[b] and 2000). In the case of *Nerine*, for example, both the apical and middle parts of the peduncle gave approximately equal regeneration while regeneration from the basal parts was less (PIERIK & STEEGMANS, 1986). The upper half of the *Hippeastrum* flower stem also produced more shoots (ALDERSON & RICE, 1986). In other studies involving *Nerine* sp., the basal half of the floral stem produced more shoots per explant (ALDERSON & RICE, 1986). It therefore appears that the response of the explant is influenced by the physiology of the parent tissue and is possibly related to endogenous levels of hormones (ALDERSON & RICE, 1986).

Materials and methods

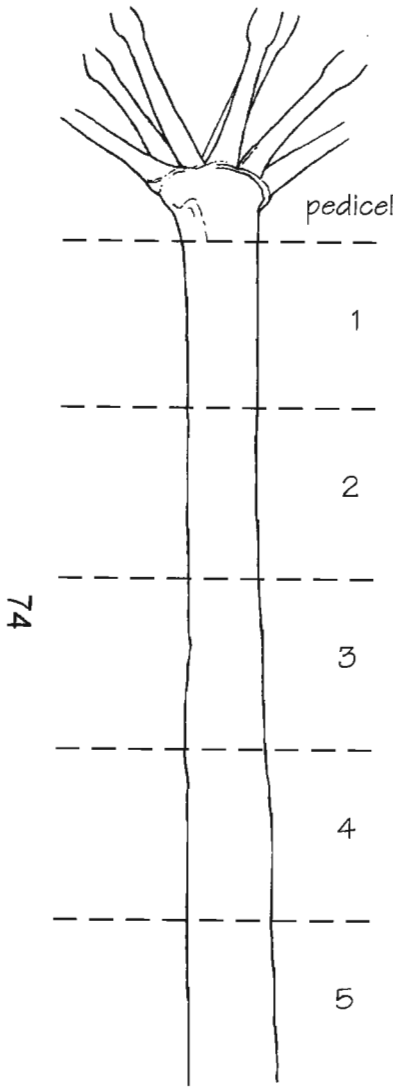
Serial sections were made from the apex of the peduncle (position 1), including the pedicel-peduncle junction, to the base of the peduncle (position 5) (Figure 4.2) . Disks were cultured on M&S medium with 2 mg ℓ^{-1} BA and 0.5 mg ℓ^{-1} NAA.

Results

Shoots formed at the pedicel-peduncle junction at the apex of the flower stem (Figure 4.2) but in only 6% of the explants. No growth was recorded for explants taken from positions 1 - 5.

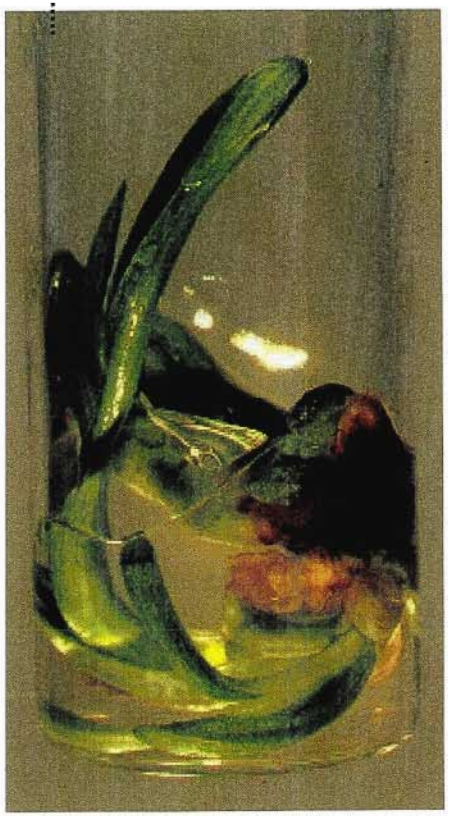
Discussion

The production of shoots was only recorded for explants excised at the junction of the pedicel and peduncle of *C. moorei*. The origin of shoots at the pedicel-peduncle junction, has, in other instances, been linked to actual flower meristems or floral axillary buds (GEORGE, 1993). In onion, it appeared that the shoots arose adventitiously over the entire receptacle surface (DUNSTAN & SHORT, 1977). Plantlets differentiated in a similar fashion in *Brodiaea*, *Ornithogalum dubium* (ZIV & LILIEN-KIPNIS, 1997[b]), *Nerine* x mansellii and *N. sarniensis* (LILIEN-KIPNIS *et al.*, 1990; ZIV & LILIEN-KIPNIS, 2000) but occurred alongside callus, which was interspersed with nodular meristematic tissue, in the case of *Nerine* (LILIEN-KIPNIS *et al.*, 1992). The hormones 2,4-D and BA were used to initiate growth of the nodular tissue which was subsequently used for the induction of somatic embryos and proliferation



position in the peduncle	5 cm	10 cm	15 cm	20 cm
shoots		(no response) x	x	x
1	x	x	x	x
2	x	x	x	x
3	x	x	x	x
4	x	x	x	x
5	x	x	x	x

peduncle age



shoots at the pedicel-peduncle junction

Figure 4.2: The effect of explant age and position on shoot production from peduncles

of plantlets in liquid culture. *Hippeastrum* pedicels are also known to produce shoots (ALDERSON & RICE, 1986; FOUNTAIN & O'ROURKE, 1980) while only the tissue between the pedicels of *Allium ampeloprasum* was found to be regenerative. Polarity effects have even been recorded within the pedicel. Explants excised nearest the receptacle of *Lilium longiflorum* produced five times as many buds than those towards the proximal end (LIU & BURGER, 1986; cited by GEORGE, 1993). De Bruyn *et al.* (1992), however, report that is was mainly those disks that were taken just underneath the attachment of the flower stems that regenerated new plants of *Amaryllis belladonna*. Highly regenerative explants of *Gladiolus* are also to be found below the expanding florets which are still enclosed by the bracts (ZIV & LILIEN-KIPNIS, 1997[b] and 2000). It is the stalks of *Brodiaea* and *Ornithogalum dubium* that are highly regenerative (ZIV & LILIEN-KIPNIS, 1997[b]). Here too, position effects were noted. In *Hippeastrum*, for example, more shoots regenerated when explants were taken from the upper half of the stems (ALDERSON & RICE, 1986). A gradient response, toward the shoot apex, was also observed in the stems of *Alstroemeria* (LIN *et al.*, 1998). The youngest explants near the shoot apex gave the highest response in terms of percentage shoot regeneration and in the number of shoots per regenerating explant. Pierik and Steegmans (1986) compared the regeneration of peduncle disks taken from the top, middle and base of the floral stem of *Nerine bowdenii* and concluded that regeneration from the base was less than that from either the middle or top. The rate of differentiation from the scapes of *Narcissus pseudo-narcissus* also increased from the base to the apical parts (HYANGYOUNG *et al.*, 1997). Fountain and O'Rourke (1980), on the other hand, report that 'explant productivity' increased towards the proximinal end of the flower stem and that the pedicel tissue was more productive than the ovary tissue in producing callus and plantlets. In asparagus, explants taken from the region distal to the apex produced callus more readily than tissues nearer the apex (TAKATORI *et al.*, 1968). The basal and first nodal segments of the floral stem of tulip were also found to be more reactive than the upper nodes (RICE *et al.*, 1983). The best explants for regeneration from *Narcissus* peduncles are those closer to the basal plate (ZIV & LILIEN-KIPNIS, 1997[b] and 2000). The effect of polarity on the type of morphogenic response was shown in *Gladiolus* inflorescence stalks. Segments taken from the basal ends produced callus and roots but at the distal end formed buds and cormlets (ZIV *et al.*, 1970).

As with other bulbous species like *Amaryllis belladonna*, *Gladiolus*, *Hippeastrum*, *Nerine* and tulip, shoot regeneration from floral stems of *C. moorei* was influenced by the position of the explant in the mother plant. Perhaps this can be explained by differences in endogenous levels of hormones. Auxin promotes the efflux of H^+ in basal cells and so maintains an electrical potential difference between the shoot apex (positive) and base (negative). This, in turn, is thought to maintain and reinforce the transport of auxin in a polar direction (RAVEN, 1979; cited by GEORGE, 1993). Saniewski and Kawa-Miszczak (1997; cited by

BOONEKAMP, 1997) also showed that auxins are transported basipetally during stem elongation and that transport in this direction was, in fact, necessary for elongation. Other studies have shown that there is a significant increase in the levels of GA_3 along the length of the stem towards the flower e.g. tulip (WRIGHT, 1981; cited by ALDERSON & RICE, 1986). Basal explants are therefore less desirable. In asparagus, they show increasing woodiness and contamination by endogenous micro-organisms (TAKATORI *et al.*,1968), such that explants from an apical position are preferred.

EXPLANT POLARITY

Introduction

Polarity effects have been observed for the inflorescence stalks of *Narcissus, Gladiolus, Asparagus* and *Amaryllis* hybrids (HUGHES, 1981) as well as for *Hippeastrum* (SEABROOK & CUMMING, 1977; ALDERSON & RICE, 1986), *Nerine* (ALDERSON & RICE, 1986; PIERIK & STEEGMANS, 1986) and *Ornithogalum dubium* (ZIV & LILIEN-KIPNIS, 2000). Reversed polarity i.e. with the morphological base away from the medium, is in some instances an absolute requirement for shoot induction (HUGHES, 1981). This is possibly related to polar transport of auxin (SEABROOK *et al.*, 1976). Reversed polarity may also lead to greater shoot induction (HUGHES, 1981) but will prevent shoot regeneration in species like *Hippeastrum* and *Nerine* (ALDERSON & RICE, 1986).

Materials and methods

Peduncles 16 - 32 cm in length were removed from the plant before anthesis. These were sterilized in 2% NaOCℓ for 15 minutes. Explants, 2 mm thick, were inoculated onto M&S medium with the morphological base either on or away from the medium i.e. with normal or reversed polarity.

Results

Callus formed on the epidermal surface of explants placed in both orientations. The number of explants that responded in this manner was, however, limited to two for both treatments. Whereas explants with their morphological base in the medium developed callus that was crystalline and green, explants with reversed polarity had granular and green callus.

Discussion

The orientation of the explants of *C. moorei* did not appear to affect their regeneration ability. Floral stems of *Nerine bowdenii* (PIERIK & STEEGMANS, 1986) were cultured with their basal ends on the medium since inverted scapes of nerine as well as hippeastrum cannot regenerate shoots (ALDERSON & RICE, 1986). Earlier, Seabrook and Cumming (1977; cited by ALDERSON & RICE, 1986) reported that inverted scapes were more responsive. It is also advantageous to invert floral stems of asparagus (TAKATORI *et al.*,1968), *Narcissus* (SEABROOK *et al.*, 1976; KOZAK, 1991) and *Gladiolus* (ZIV *et al.*, 1970) in order to obtain a morphogenic response. Inflorescence discs of *Gladiolus* showed rapid morphogenesis when placed in an inverted position compared to discs placed in their natural orientation. The latter developed callus and roots from their basal ends. Buds, however, developed from the distal end of inverted discs and after placing them with their basal ends in the medium produced clusters of cormlets during subsequent culture (ZIV *et al.*, 1970). Thus, in some instances, growth and morphogenesis may be influenced by the way the explant is orientated on the medium. According to George (1993) the effects may be due entirely to polarity or to the availability of nutrients and hormones to those tissues that are competent.

THE CULTURE ENVIRONMENT: LIGHT

Introduction

The effect of light on bulblet regeneration from floral stem explants was studied in *Nerine bowdenii* and found to be less effective in promoting bulblet formation than those cultures that were placed in continuous darkness (PIERIK & STEEGMANS, 1986). Yet there are other reports that show that light is necessary for bulblet production (RICE, ALDERSON & WRIGHT, 1983; cited by ALDERSON & RICE, 1986).

Materials and methods

Peduncles 10 - 23 cm in length were sterilized in 2% NaOCℓ for 15 minutes and then placed on the M&S medium. Cultures were grown in the light (70.7 $\mu mol.m^{-2}.s^{-1}$) with a 16:8 hour light:dark cycle or in continuous darkness.

Results

No cell division was initiated in explants grown in continuous darkness. Explants grown in the light produced only a wound callus. Of these, 4% responded by producing callus or shoots.

The callus was nodulated and appeared on the epidermal surface. Translucent to green shoots also formed around the circumference of the peduncle disks.

Discussion

Callus and shoots developed on *C. moorei* peduncle explants grown in the light. In *Narcissus pseudo-narcissus*, dark treatment did not promote adventitious shoot differentiation (HYANGYOUNG *et al.*,1997). Those of tulip also showed enhanced bulblet development in the light compared to the dark (RICE, ALDERSON & WRIGHT, 1983; cited by ALDERSON & RICE, 1986). When inflorescence explants of *Bowiea volubilus* are cultured in the dark, however, plants regenerated and formed bulbs (HANNWEG *et al.*, 1996). Pierik and Steegmans (1986) also reported that adventitious bulblet formation was better in the dark and that the floral stem explants were particularly sensitive to light. Light is important for the normal growth of green shoots and plantlets since it induces chlorophyll formation. It is thus necessary for adventitious bud development, but may not be essential for unorganised cultures, for example, which grow especially well in the dark (GEORGE, 1993).

MAINTENANCE OF CALLUS GROWTH AND INDIRECT ORGANOGENESIS

Introduction

Indirect organogenesis from inflorescence-derived callus has been reported for a number of bulbs. This may be achieved in several ways, namely: placing the callus on hormone-free media (HUSSEY, 1977); reducing the level of auxin (HUSSEY, 1975, 1978 and 1980); changing the source of auxin or hormone used in the inductive medium (HUSSEY, 1975) and maintaining the callus in the dark (SEABROOK *et al.*, 1976).

Materials and methods

Callus that formed during the growth induction phase on pedicel explants was subcultured and maintained on M&S medium with 5 mg ℓ^{-1} NAA and 5 mg ℓ^{-1} BA.

The effect of light and of reducing the concentration of auxin were investigated by placing the callus on 0, 0.1, 1 and 5 mg ℓ^{-1} NAA together with 5 mg ℓ^{-1} BA either in the light (16:8 light:dark cycle with PAR measuring 13.3 $\mu mol.m^{-2}.s^{-1}$) or dark.

Results

Callus that was subcultured and maintained on M&S medium with 5 mg ℓ^{-1} NAA and 5 mg ℓ^{-1} BA grew slowly. Two lines were obtained which differed in pigmentation. One line was creamy-white and the other brown.

New callus growth occurred only in the dark (Figure 4.3). This was visibly whiter than the older callus. Although callus production was observed over all concentrations of auxin tested, the percentage response was best in the treatment that contained no auxin (Table 4.1). Shoots never developed from the callus.

Table 4.1: The effect of hormones and light on the % callus production

NAA concentration (mg ℓ^{-1})	Light	Dark
0	0	100
0.1	0	20
1	0	20
5	0	40

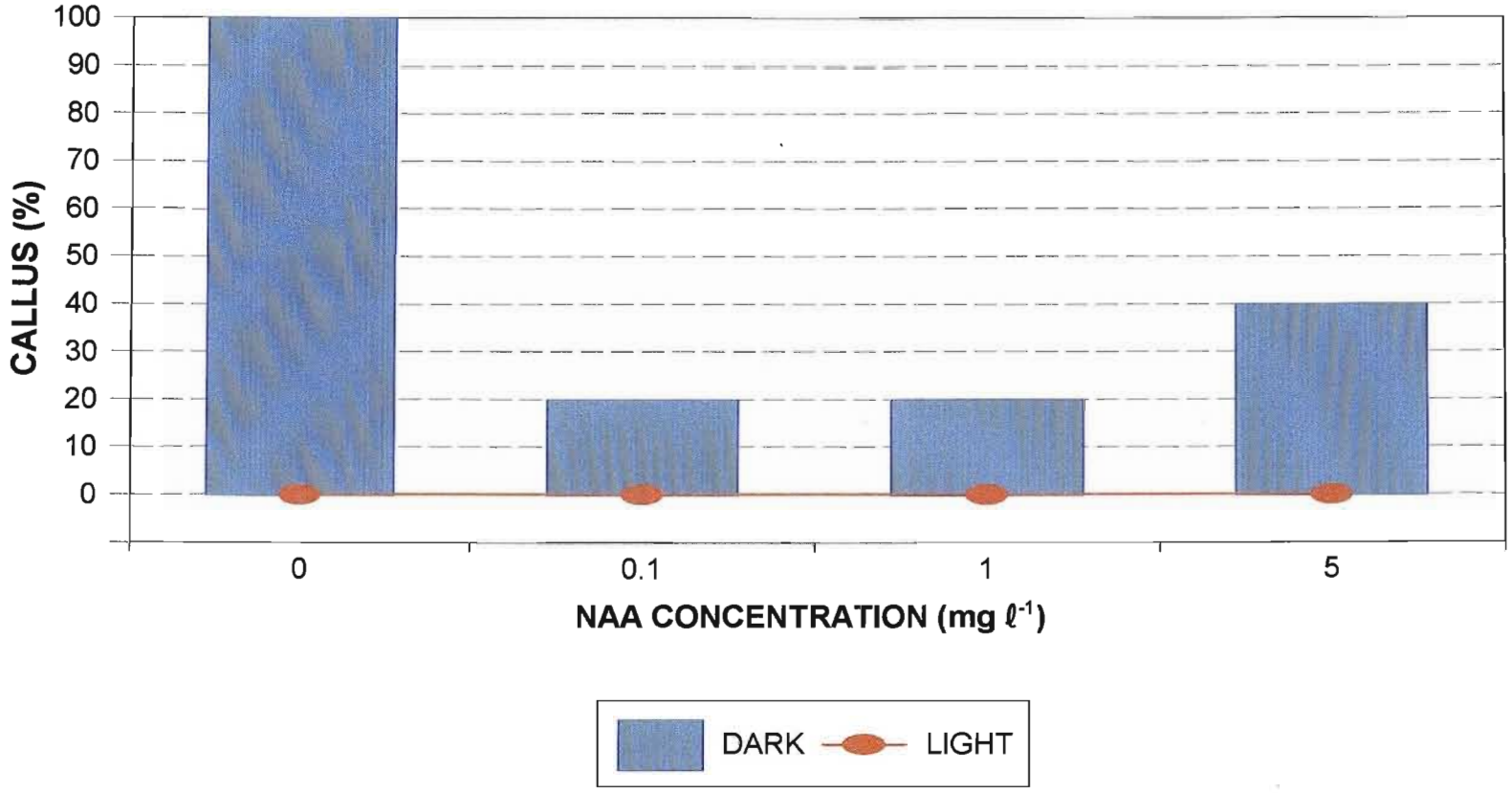


Figure 4.3: The effect of hormones and light on callus production

Discussion

The growth of callus on auxin and cytokinin supplemented media was generally poor. This is possibly related to inhibitory effects induced under high light intensities (GEORGE, 1993). In fact, subsequent tests showed that no new callus growth was possible in the light but only in the dark. Seabrook *et al.* (1976) also concluded that it was better to maintain *Narcissus* callus on 1 mg ℓ^{-1} 2,4-D in the dark compared to NAA. Incubation in the dark is also reported for *Alstroemeria* callus (GONZALEZ-BENITO & ALDERSON, 1992). There are several possible causes of light-inhibited callus growth, including: (1) the inhibition of DNA replication when light destroys vitamin B_{12}; (2) an increase in the production of phenolics; (3) the destruction of cytochrome oxidase; (4) the enhanced biosynthesis of gibberellins and (5) the inhibition of cytokinin synthesis (GEORGE, 1993). Furthermore, starch accumulates in the dark, which might explain why it is necessary to first place explants in the dark to effect indirect organogenesis, especially in the families Iridaceae and Liliaceae (HUGHES, 1981).

The growth of *C. moorei* callus was maintained on media containing both auxins and cytokinins and even in media from which NAA had been excluded. Other workers report the growth of callus on LS media which includes both 2,4-D and NAA in addition to BA (STANILOVA *et al.*, 1994) or when both 2,4-D and BA are included (LILIEN-KIPNIS *et al.*, 1992). Otherwise, auxins such as NAA and 2,4-D, are used alone (HUSSEY, 1978).

Although most calli responded positively to a reduction in the level of NAA, new callus growth was never accompanied by shoot production. In other cases, indirect organogenesis may be achieved by transferring the callus to media containing less auxin than is needed to maintain callus growth (HUSSEY, 1975 and 1978; LILIEN-KIPNIS *et al.*, 1992) and even to hormone-free media e.g. lily and *Freesia* (HUSSEY, 1977). Removal of embryogenic cell cultures to hormone-free media may also stimulate somatic embryogenesis in *Hemerocallis* (SMITH & KRIKORIAN, 1991). Sellés *et al.* (1997) found that, by adding 0.5 mg ℓ^{-1} BA to the medium, shoot-buds regenerated from the callus surface. Plantlet regeneration from callus, in *Alstoemeria*, was also possible after transfer to media containing BA (GONZALEZ-BENITO & ALDERSON, 1992). In other instances, an increase in the cytokinin to auxin ratio is stimulatory (SEABROOK *et al.*, 1976) e.g. *Narcissus* (HUSSEY, 1980). Lilien-Kipnis *et al.* (1992) found that somatic embryogenesis could be achieved by removing paclobutrazol (PAC) from the induction medium during the maturation phase. Like other "anti-gibberellins", PAC is able to stimulate embryogenesis if it is inhibited by gibberellic acid (GEORGE, 1993). The lack of any organogenic response from callus is commonly encountered in ornamental plants and has been attributed to a decline in regeneration potential as callus ages. Furthermore, the regenerative capacity of callus may depend on the hormones used in the inductive medium (HUSSEY, 1977). For example, callus that was induced and grown on NAA

differentiated plantlets more readily than callus induced by 2,4-D, except in the case of *Narcissus* (HUSSEY, 1975). The use of callus to propagate *C. moorei* is thus limited by the fact that it is difficult to produce in culture; that subsequent growth is slow and because indirect organogenesis could not be achieved.

In general, the regenerative response of *C. moorei* peduncles was poor. Blake and Eeuwens (1982; cited by GEORGE, 1993) could induce coconut flower meristems to form "shootlets" in an unshaken liquid medium. They report that the response was superior to that observed in either shaken cultures or on agar. This could, perhaps, remedy the problem for *C. moorei* peduncles.

Of the few shoots that were produced from the pedicel-peduncle junction, none developed bulblets. Pierik and Steegmans (1986), however, reported that bulblet formation in *Nerine bowdenii* was easily achieved. This also appears to be true of *Hippeastrum* as shoots that were left in culture for three months and more and without any additional treatments developed bulblets (ALDERSON & RICE, 1986). In the case of tulip, bulblets developed from shoots after approximately one year without subculture. Perhaps bulblets could be induced by cold treatment or exposure to gibberellins as for tulips (ALDERSON *et al.*, 1983; cited by PIERIK & STEEGMANS, 1986). Alderson and Rice (1986) also reported that increasing the levels of sucrose to 6% resulted in a better bulbing response, while in onion, high humidity and hormone treatments were successfully used to induce bulbils from inflorescence explants (THOMAS, 1972; cited by CRISP & WALKEY, 1974).

CONCLUSIONS

Although peduncles of *C. moorei* are capable of producing shoots, this appears to be influenced by a complex of factors including, the age of the floral stem, the position of the explants along the length of the stem and hormonal factors. They do not regenerate shoots as readily as twin-scales or undergo spontaneous bulbing. Their only advantage is that they provide an alternative source of regenerative material during the flowering period which would alleviate the problem of destructible bulb harvesting.

Regeneration from twin-scales: Factors affecting growth and morphogenesis

INTRODUCTION

Twin-scales of *C. moorei* readily produced shoots. Those of other amaryllidaceous species are equally responsive in culture. But for some genotypes it is, in fact, highly desirable to induce direct bulblet formation (ZIV, 1997). Benefits include: the prevention of hyperhydricity; the elimination of a rooting stage and the need for hardening-off and; a shorter bulb production period (ZIV, 1997). Bulblets are, therefore, generally regarded as the final product in micropropagation schemes involving bulbous plants. In addition, they have significant advantages for transplanting and survival *ex vitro* as well as for long term storage or shipment (ZIV, 1997) since they can be sown like seeds at any time of the year.

The development of bulblets *in vitro* has been studied in many commercially important bulbs. Factors that affect the development of bulblets are: explant position within the parent bulb; explant size and polarity; sucrose concentrations and levels of plant growth regulators; charcoal; light and temperature. Particular attention has been paid to the growth of explants under growth-limiting conditions such as a medium with a high osmotic value (GINZBURG & ZIV, 1973) since these are instrumental in triggering the formation of a resting organ. Tubering in potatoes, for example, occurs in most senescent cultures (HUSSEY & STACEY, 1981). Tissue-dependent factors and other environmental effects were examined in an effort to produce bulblets for hardening-off and planting out.

EXPLANT POSITION

Introduction

Differences in productivity for inner and outer scales - referred to as position effects - have been recorded for explants excised from bulbs (HANKS, 1985; cited by HANKS *et al.*, 1986) and linked to the physiological age of the organ (VAN AARTRIJK & VAN DER LINDE, 1986) and the distribution of carbohydrates (HANKS *et al.*, 1986). The youngest twin-scales are located in the centre of the bulb while the older scales are found towards the outside. For some bulbs the number of protuberances produced is greatest for the inner scales compared to the outer scales. These also readily form bulbs. In *Lilium*, for example, the internal scales form bulbs more frequently than the outer scales (TAKAYAMA & MISAWA, 1980). Another advantage of using inner scales is that they are relatively free of contaminants unlike the

outer, older bulb tissue. However, outer scales develop bulbs more frequently when the twin-scales consist of one thick outer and one thin inner scale e.g. *Hippeastrum hybridum* (HUANG *et al.*, 1990). Similar results have been achieved with *Narcissus* and *Hyacinthus* twin-scales. This can be explained by the fact that the explants contain larger amounts of food reserves (HUANG *et al.*, 1990). Slabbert *et al.* (1993), however, found that the average number of plantlets was greater for the middle part of the bulb in *Crinum macowanii*.

Materials and methods

Twin-scales were divided into three groups according to their position in the parent bulb - inner (position 1), middle (position 2) and outer (position 3) (Figure 3.2). These were placed under optimal growth conditions.

Results

Although frequently contaminated, explants from the outer parts of the bulb always produced shoots. The response from the inner twin-scales was comparatively poorer (Table 4.2). Bulblets formed in the axes of twin-scales regardless of their position in the parent bulb. The best response, however, was noted for twin-scales from position 2, where 1.2 bulblets developed in 83% of the twin-scales (Table 4.2). These bulblets were, on average, slightly larger than those for positions 1 and 3 although this difference was not statistically significant (Table 4.2).

Table 4.2 : The effect of explant position on bulblet regeneration and growth (after 17 weeks)

Explant Position	Shoots (%)	Bulblets (%)	Mean number bulblets / explant	Mean bulblet diameter (mm)
Position 1 (Inner scales)	17	56	1.0[a]	7.2[a]
Position 2 (Middle scales)	8	83	1.2[a]	7.3[a]
Position 3 (Outer scales)	33	67	1.0[a]	7.0[a]

Different letters show significant differences between treatments at the 5% level (ANOVA).

Discussion

The outer, older twin-scales of *C. moorei* were frequently contaminated: a condition which is also common amongst *Narcissus* bulbs (SQUIRES & LANGTON, 1990). Sterile explants from this position, however, produced shoots more frequently than explants from other positions within the bulb. Although regeneration from scales of *Hyacinth* cultivars was not influenced by the position in the parent bulb, bulblet growth (weight) was better in the outer scales (PIERIK & RUIBING, 1973). This is in contrast to bulbs like *Hippeastrum hybridum* (HUANG *et al.*, 1990) and *Lilium* (TAKAYAMA & MISAWA, 1980) where the number of protuberances was found to be greatest for the inner scales. Higher regeneration activity in the inner scales of the bulb compared to the outer bulb scales is also reported for a number of *Lilium*, *Hyacinthus orientalis*, *Gladiolus* and *Narcissus* cultivars (CHURIKOVA & BARYKINA, 1995). Inner scales are preferred for reasons other than their regenerative ability, and that is because the outer, older bulb tissue has a higher contamination frequency (SQUIRES & LANGTON, 1990). Twin-scales from position two were superior in terms of bulblet development. Those from the middle parts of the bulb also produced the most plantlets in *Crinum macowanii* (SLABBERT *et al.*, 1993) and *Narcissus* sp. (HANKS, 1986; cited by SLABBERT *et al.*, 1993). Adventitious bulbil production in *Narcissus* following twin-scaling, decreased centripetally but was lowest in the outermost twin-scales (HANKS *et al.*, 1986). Larger bulblets developed in twin-scales removed from the centre of the bulb. This may be due to the presence of higher food reserves since the outer scales are thinner and less fleshy. Myodo and Kubo (1952; cited by TAKAYAMA & MISAWA, 1980) reported that there were differences in the chemical constituents of inner and outer bulb scales, especially in the level of soluble nitrogen and sugar. Internal bulb scales of *Lilium* had lower sugar levels. In *Narcissus*, the inner scales were found to be rich in insoluble sugars while those that were of intermediate position were highest in reducing, total soluble and insoluble sugars (HANKS *et al.*, 1986; cited by HANKS, 1993). This might explain why more bulblets formed in twin-scales from position two in *Crinum moorei*.

Owing to the different growth characteristics of twin-scales from different positions within the parent bulb and the high contamination frequency of the outer twin-scales, it may be worthwhile to use twin-scale from all positions, combining the advantages of each.

EXPLANT SIZE

Introduction

Bulblets form nearest the vascular bundles in twin-scales of the Amaryllidaceae and Liliaceae (YANAGAWA & SAKANISHI, 1980; cited by HUANG *et al*, 1990). In amaryllidaceous species, the vascular bundles run along the abaxial side of the scales and the regenerative capacity is thus higher than that on the adaxial side (HUANG *et al.*, 1990). As bud formation in excised tissues is, to a large extent, dependent on the presence of vascular tissue (HUANG *et al.*, 1990), the size of twin-scales may determine their ability to produce buds. Small explants generally have low survival rates in culture (HUGHES, 1981). By increasing the length of the scales, bulblet regeneration increases in species like *Hippeastrum hybridum* (HUANG *et al.*, 1990) and *Eucharis grandiflora* (PIERIK *et al.*, 1983). Bulblet weight also increases when larger (longer and wider) explants are used (PIERIK & IPPEL, 1977). Thus, explant size is critical for survival, regeneration potential and ultimate bulblet size.

Materials and methods

In order to determine the effect of twin-scale size on bulblet regeneration, twin-scales were cut to different widths, namely 3 mm, 5 mm, 6 mm and 10 mm. Each of these ranged in length from 5 - 15 mm. The explants were placed on a full strength M&S medium without hormonal supplements. Cultures were incubated at 25°C.

Results

Most (69%) 5 mm twin-scales produced bulblets compared to only 44% for the narrower 3 mm twin-scales. After 19 weeks, the average bulblet diameter was slightly smaller, at 4.5 mm, for the 5 mm twin-scales compared to 4.7 mm for the 3mm twin-scales. The effect of increasing the length and width of the explants is tabulated below (Table 4.3). The largest twin-scales (10 mm wide) and those measuring 6 mm x 10 mm were all contaminated. Bulblets developed in all the remaining size classes, except the smallest which measured 3 x 5 mm. Bulblet regeneration was highest in the 6 x 15 mm twin-scales but the 3 x 15 mm twin-scales produced more bulblets (1.8) per explant (Table 4.3). The twin-scales which were 6 mm wide yielded larger bulblets compared to the smaller 3 mm twin-scales (Table 4.3).

Table 4.3 : The effect of twin-scale size on bulblet regeneration (after 19 weeks)

Twin-scale size (mm) Diameter x length	Shoots (%)	Bulblets (%)	Mean number of bulblets / explant	Mean bulblet diameter (mm)
3	11	44	1.0^{ab}	4.7^{ab}
5	23	69	1.0^{b}	4.5^{b}
3 x 5	100	0	-	-
3 x 10	33.3	66.7	1.0^{ab}	7.0^{ab}
3 x 15	16.66	83.33	1.8^{a}	6.6^{ab}
6 x 5	33.3	66.7	1.0^{ab}	9.5^{ab}
6 x 10	All contaminated			
6 x 15	0	100	1.2^{ab}	9.0^{a}
10 x 5	All contaminated			
10 x 10				
10 x 15				

Different letters show significant differences between treatments at the 5% level (ANOVA).

Discussion

More bulblets developed amongst the 5 mm twin-scales compared to the narrower, 3 mm twin-scales. This suggests that the size of the twin-scale influences the regeneration potential and subsequent development of the bulblets. In fact, no bulblets formed in the smallest twin-scales measuring 3 x 5 mm. Smaller explants of *Iris hollandica* were also reported to show reduced regeneration (VAN DER LINDE *et al.*, 1988). In Lily, larger bulblets regenerated on large explants (LANGENS-GERRITS *et al.*, 1997).

The length of the twin-scales appears to be important for bulblet development in *C. moorei*: a phenomenon which, previously, was reported in other amaryllidaceous species like *Hippeastrum* and *Eucharis*. Twin-scales that were shorter than 10 mm did not produce bulblets in *Hippeastrum hybridum* (HUANG *et al.*, 1990). According to Okubo *et al.* (1990), protuberances were observed, but bulblet development was poorer for scales less than 10 mm. By increasing the length of scales, bulblet regeneration was increased in *Eucharis grandiflora* (PIERIK *et al.*, 1983). In *Hyacinth* cultivars, the speed of regeneration and regeneration percentage, as well as the number of bulblets per explant and their weight, were found to be proportional to the lengths of the explants (PIERIK & RUIBING, 1973). An increase in scale length thus strongly enhanced the growth of regenerating bulblets. In *Hyacinthus orientalis*,

the number of bulblets produced was found to be proportional to the length of the explants (PIERIK & POST, 1975). A higher length to width ratio proved to be essential for good regeneration and bulblet growth (PIERIK & POST, 1975). Bulblet weight was also greatly increased using longer twin-scales (PIERIK & POST, 1975).The length of the twin-scale only slightly influenced the number of bulblets produced in *Nerine*, but, bulblet weight increased significantly when longer explants were used (PIERIK & IPPEL, 1977). The importance of twin-scale length may be related to the amount of food reserves in the scale (HUANG *et al.*, 1990; OKUBO *et al.,* 1990). Pierik and Ruibing (1973) suggest that the distal parts of the scales play an important role as sources of carbohydrates and other substances.

Larger bulblets developed in *C. moorei* when the diameter of the twin-scale exceeded 3mm. Increasing the width of *Nerine* twin-scales from 0.3 - 1 cm, also, was found to lead to an increase in bulblet formation and growth (PIERIK & IPPEL, 1977).

Twin-scales may have either a stimulatory or inhibitory effect on growth (VAN AARTRIJK & VAN DER LINDE, 1986) depending on their size. Such differences in response are thought to be related to the amount of wound damage which stimulates cell division (VAN AARTRIJK & VAN DER LINDE, 1986). Larger twin-scales generally produce more bulblets e.g. *Nerine* (PIERIK & IPPEL, 1977) and *Eucharis grandiflora* (PIERIK *et al.*, 1983), perhaps because they have more vascular bundles. Bud formation in excised tissues is closely related to the presence of vascular tissue as cells nearest the vascular tissue receive more cell division stimuli. Furthermore, vascular connections eventually form between the bulblet primordia and the parent tissue (HUANG *et al.*, 1990) which might explain why the presence of the explant during the entire regeneration period was found to be necessary in Lily (LANGENS-GERRITS *et al.*, 1997). Langens-Gerrits *et al.* (1997) propose several hypotheses for the regeneration of large bulblets on large explants. Firstly, more nutrients are taken up by large explants as they have a greater surface area in contact with the medium. Secondly, the explants themselves contain compounds necessary for growth, which are lacking in the medium. And, thirdly, large explants have more "processing" enzymes which are available for nutrient conversion. Although they came to the conclusion that the contact area of the explant with the medium strongly influenced bulblet growth and final weight, this cannot explain the superior growth in larger *C. moorei* explants since all were placed on their sides and not with their adaxial or abaxial surfaces in contact with the medium.

EXPLANT POLARITY

Since the position of explants affects the regeneration potential of bulb scales, it is necessary to orientate the explants correctly on the medium (VAN AARTRIJK & VAN DER LINDE, 1986). Several workers report that inverted explants yield more plantlets per explant (PIERIK

& RUIBING, 1973; PIERIK & IPPEL, 1977; JACOBS *et al.*, 1992). Whether the scales are inverted or placed with the basal plate in the medium may, in other cases, make no difference to the number of plantlets produced (DE BRUYN *et al.*, 1992). It is often preferable though, to insert the basal plate in the medium to avoid desiccation (DE BRUYN *et al.*, 1992). Also, improved regeneration has been achieved in species like *Eucharis grandiflora* (PIERIK *et al.*, 1983) when the scales are not inverted.

In this study, the twin-scales were placed on their sides. Not only was this more practical, since the twin-scales could not fall over, but it also avoided the problem of poor oxygen supply at the basal plate end. This has been reported for *Hyacinth* cultivars (PIERIK & RUIBING, 1973) where the scales were inserted in the medium to a depth of about half their length. Preliminary studies with *C. moorei* twin-scales also showed that scales with their dorsal (adaxial) surface toward the medium readily turned black.

SUCROSE CONCENTRATION

Introduction

Sucrose is the preferred carbon source for the cultivation of many horticultural crops *in vitro* (HUGHES, 1981) and is essential for shoot production. In some species the absence of sucrose is marked by the lack of differentiation and concomitant storage organ development (TAKAYAMA & MISAWA, 1979; DE BRUYN & FERREIRA, 1992). Sucrose stimulation of bulbing has been reported for a number of bulbous crops e.g. onion, *Lilium*, *Tulipa*, *Iris* and *Gladiolus* (TAEB & ALDERSON, 1990; CHOW *et al.*, 1992[a]; DE BRUYN & FERREIRA, 1992). This is achieved at optimum concentrations of between 3 and 10%, which is much higher than concentrations used for other crops (VAN AARTRIJK & VAN DER LINDE, 1986). An increase in the level of sucrose improves the size of micropropagated bulblets (TAKAYAMA & MISAWA, 1979; VAN AARTRIJK & BLOM-BARNHOORN, 1980; SQUIRES *et al.*, 1991; CHOW *et al.*, 1992[b]; MARINANGELI & CURVETTO, 1997) which is critical for subsequent *ex vitro* stages (MARINANGELI & CURVETTO, 1997). It may also stimulate root formation (TAKAYAMA & MISAWA, 1979; HUGHES, 1981; LILIEN-KIPNIS *et al.*, 1992).

Materials and methods

The effect of sucrose on both shoot and bulblet development was assessed by including sucrose in the medium at concentrations of 0, 2, 4, 6, and 8 g ℓ^{-1}. Twin-scales were placed under optimal growth conditions.

Results

The effect of sucrose concentration on shoot regeneration was studied separately from its effects on bulblet development. Shoots developed in all treatments with the highest percentage regeneration at levels of 4% sucrose. The mean length of shoots varied between treatments. Those growing on media supplemented with 2% sucrose were the longest, at 8 mm, after 10 weeks (Table 4.4). This differed significantly from those grown on 8% sucrose.

Table 4.4: The effect of sucrose concentration on shoot initiation and growth (after 10 weeks)

Sucrose (%)	Shoots (%)	Mean number shoots / explant	Mean shoot length (mm)
0	21	1.0^{a}	5.3^{ab}
2	67	1.0^{a}	8.3^{a}
4	100	1.0^{a}	7.3^{ab}
6	60	1.1^{a}	4.9^{ab}
8	55	1.2^{a}	3.3^{b}

Different letters show significant differences between treatments at the 5% level (ANOVA).

Twin-scales that were placed on 0% sucrose generally responded poorly with none producing bulblets (Table 4.5). The greatest percentage bulbing (40%) was recorded for twin-scales placed on 6% sucrose. However, they produced significantly fewer bulblets per explant compared to the 4% sucrose treatment. Bulblets grown on 2% sucrose attained a maximum average diameter of 8.3 mm.

Table 4.5: The effect of sucrose concentration on bulblet regeneration and growth (after approximately 33 weeks)

Sucrose (%)	Bulblets (%)	Mean number bulblets / explant	Mean bulblet diameter (mm)
0	0	-	-
2	18	1.0^{b}	8.3^{a}
4	14	2.33^{a}	6.6^{a}
6	40	1.2^{b}	6.2^{a}
8	14	1.0^{b}	6.7^{a}

Different letters show significant differences between treatments at the 5% level (ANOVA).

Discussion

Twin-scales of *C. moorei* responded poorly, if at all, to the lack of sucrose in the basal medium. Thus, as in other geophytes e.g. *Gladiolus* (DE BRUYN & FERREIRA, 1992), sucrose is essential for shoot production. This is because it regulates the rate of regeneration and organogenesis (first reported in tobacco) (TAEB & ALDERSON, 1990) and is the main sugar for starch accumulation, which occurs in regions that ultimately form shoots (VAN AARTRIJK & BLOM-BARNHOORN, 1980). Culture on a sugar medium also increases, not only the photosynthetic potential of the tissues, but the resistance to high light intensities of plantlets grown *in vitro* (TICHA *et al.*, 1998).

Shoot proliferation occurred at levels of 4% sucrose. This is comparable to the results achieved for *Narcissus* cultivars (CHOW *et al.*, 1992[b]) where 3% sucrose stimulated the formation of shoots; with no significant improvement at higher concentrations. Shoot production in *Crinum* 'Ellen Bosanquet' (ULRICH *et al.*, 1999) and in tissue-cultured yams was also found to be retarded when high sucrose concentrations of 18% (ULRICH *et al.*, 1999) or 8 - 10% were used (NG, 1988). Since bulb scale tissue contains large amounts of starch, relatively low levels of exogenous sucrose are required for shoot initiation (VAN AARTRIJK & BLOM-BARNHOORN, 1980). The growth of shoot clumps of *Narcissus confusus* was, however, best with 9% sucrose (SELLÉS *et al.*, 1997) although higher concentrations also led to a reduction in growth.

Bulbing of *C. moorei* shoots requires higher than normal levels of sucrose as do those of several other bulbous crops. Takayama and Misawa (1979), for example, noted that organ formation from *Lilium* bulb scales was stimulated by an increase in sucrose. In *Narcissus* cultivars (HOSOKI & ASAHIRA, 1980) and tulips (TAEB & ALDERSON, 1990), bulblet formation was promoted using concentrations of between 3 and 5% or even higher at 6 and 9% (LANGENS-GERRITS & NASHIMOTO, 1997). Bulb growth of *Nerine samiensis* (VISHNEVETSKY *et al.*, 1997), *Eucrosia* and *Narcissus* (ZIV & LILIEN-KIPNIS, 2000) was reportedly best at 6% sucrose. 6 g ℓ^{-1} sucrose was also beneficial for bulb growth of *Crinum* 'Ellen Bosanquet' (ULRICH *et al.*, 1999). But increasing the concentration of sucrose to levels of 6 - 9% resulted in an increase in the number of shoots forming bulbils in *Narcissus* (CHOW *et al.*, 1992[a]). Sucrose (8%) increased the number of garlic shoots that formed bulbs by as much as 86 - 90%. It also had a promotory effect on bulb diameter and the number of bulbs formed per basal plate (ZEL *et al.*, 1997). High sucrose concentrations also contributed to increased corm production of several genotypes in the Brodiaea complex (ILAN *et al.*, 1995) and in *Gladiolus* corms (DANTU & BHOJWANI, 1987; cited by ILAN *et al.*, 1995). Tuber formation in potatoes and *Dioscorea* was also promoted by increasing the concentration of sucrose in the medium (GINZBURG & ZIV, 1973; cited by ABBOTT & BELCHER, 1986).

The largest bulblets for *C. moorei* twin-scales were recorded on 2% sucrose. This is in contrast to the findings of Takayama and Misawa (1979), Van Aartrijk and Blom-Barnhoorn (1980), Squires *et al.* (1991) and Chow *et al.* (1992[a]). For in *Lilium*, increased sucrose concentrations (90g) produced an increase in bulblet fresh mass (TAKAYAMA & MISAWA, 1979; VAN AARTRIJK & BLOM-BARNHOORN, 1980; MARINANGELI & CURVETTO, 1997) and bulbil diameter increased, up to three-fold, in *Narcissus* (CHOW *et al.*, 1992[a]). Larger bulblets possibly result from the increase in total carbohydrate and starch content as a consequence of increased sucrose levels in the medium (LANGENS-GERRITS *et al.*, 1997). The explant thus acts as both a sink (growing organ) and source, that is, nutrient source for the growing bulbs (LANGENS-GERRITS *et al.*, 1997). An increase in the concentration of sucrose is also known to create a high osmotic potential in the medium. According to Ginzburg and Ziv (1973; cited by DANTU & BHOJWANI, 1995) this is a growth-limiting condition which can be exploited in the formation of potato tubers. In *Lilium* however, there is some degree of uncertainty as to whether sucrose acts through the regulation of osmotic potential or directly on internal physiological processes (TAKAYAMA & MISAWA, 1980). In the case of *Dioscorea rotundata*, sucrose effects on tuberization are thought to be linked to its effects on hormone levels (NG, 1988) in view of the fact that carbohydrates have been found to increase the efficiency of auxin transport (KRUL & COLCLASURE, 1977; cited by NG, 1988). While sucrose promotes tuber formation in potatoes, it also reduces shoot growth (ABBOTT & BELCHER, 1986). The inhibition of apical growth by sucrose may thus cause an increase in tubering (WAREING & JENNINGS, 1980). In *Gladiolus*, water stress has been shown to increase the sink capacity (ROBINSON *et al.*, 1983; cited by DANTU & BHOJWANI, 1995) of corms, leading to an increase in corm size.

In other instances, high carbohydrate levels may have little effect on bulblet regeneration e.g. saccharose and *Eucharis grandiflora* (PIERIK *et al.*, 1983) or a negative effect on the regeneration potential of twin-scales (DE BRUYN *et al.*, 1992). Vishnevetsky *et al.* (1997) reported that both bulb growth and leaf elongation were inhibited when the medium contained 9% sucrose. The inhibition of scale leaf formation in *Lilium* occurs at concentrations of 90 g ℓ^{-1} (TAKAYAMA & MISAWA, 1980) while bulblet growth is inhibited at 150 g ℓ^{-1} (TAKAYAMA & MISAWA, 1979). Hughes (1981) reports that high sucrose concentrations inhibited organogenesis and bulblet production in the Liliaceae. This may be related to the early onset of dormancy which occurs at sucrose levels of between 60 and 90 g ℓ^{-1} (SQUIRES *et al.*, 1991). On the other hand, some bulbs are unable to completely utilize sucrose in the medium e.g. *Nerine sarniensis* (VISHNEVETSKY *et al.*, 1997), which may account for the lack of improved bulb growth at higher sucrose concentrations. Carbohydrate utilization, however, can be intensified by increasing the level of phosphorus (P) in the medium. This allows for the optimal conversion of sugars to storage carbohydrates (VISHNEVETSKY *et al.*, 1997).

Sucrose is thus essential for shoot development as well as for the bulbing of *C. moorei* shoots. The latter occurs at a range of concentrations but may require higher than normal levels of sucrose to increase the frequency of bulblet development. Levels of more than 6% sucrose lead to a decrease in the formation of bulblets. Thus, an increase in the concentration of sucrose does not result in a proportional increase in the diameter of bulblets.

PLANT GROWTH REGULATORS

Introduction

The role of hormones in the regulation of storage organ formation has been studied in potatoes (PALMER & SMITH, 1969; LOVELL & BOOTH, 1967; TIZIO, 1971; cited by GINZBURG & ZIV, 1973) and bulbous plants. Not only do plant growth regulators stimulate the induction of shoots from twin-scales, but their subsequent differentiation appears, also, to be regulated by the interaction of cytokinins and auxins. This is strictly species dependent. Auxins either induce bulblet formation e.g. *Narcissus* cultivars (CHOW *et al.*, 1992[a]) or inhibit their development e.g. *Hippeastrum hybridum* (MII *et al.*, 1974). Cytokinins, alone, are used for bulblet formation in *Nerine* hybrids (CUSTERS & BERGERVOET, 1992); tuber induction in stolon tips of potato (PALMER & SMITH, 1969; cited by GINZBURG & ZIV, 1973) and in the formation of cormels in *Gladiolus* (GINZBURG & ZIV, 1973). Their use in *Narcissus* cultivars (CHOW *et al.*, 1992[a]) and *Hippeastrum hybridum* (MII *et al.*, 1974) is reported to inhibit bulblet regeneration; sometimes even leading to explant death. When used in combination, auxins and cytokinins may stimulate bulblet regeneration (TAKAYAMA & MISAWA, 1979) or inhibit their formation (STEINITZ & YAHEL, 1982; MOCHTAK, 1989; DREWES & VAN STADEN, 1994). In general, bulblet or cormlet formation occurs spontaneously in the absence of plant growth regulators (TAKAYAMA & MISAWA, 1979; STEINITZ & YAHEL, 1982; ALDERSON & RICE, 1986; STEINITZ & LILIEN-KIPNIS, 1989; DE BRUYN & FERREIRA, 1992) although this may depend on the duration and temperature of storage prior to explantation (MOCHTAK, 1989).

Materials and methods

Shoots derived from twin-scale cultures were maintained on media containing auxins and cytokinins in different combinations. NAA and BA were used in the following concentrations: 0, 0.1 and 1 mg ℓ^{-1}, in a three-by-three hormone grid. The effect of increasing the cytokinin to auxin ratio was investigated using the following concentrations and combinations of BA and NAA (mg ℓ^{-1}): 0:0, 0.5:0, 1:0, 2:0, 0:0.5, 1:0.5, 2:0.5, 0:1, 1:1, 2:1.

Results

Although adventitious shoots developed in the axes of twin-scales both in the presence and absence of plant growth regulators, the addition of hormones to the medium did not enhance the growth of the bulblets. At higher concentrations they, in fact, reduced bulblet regeneration in twin-scales and induced abnormal organogenesis (Table 4.6 and Figure 4.4). Roots were grossly enlarged and leaves vitrified. Swollen ovoid structures were also visible. These green pigmented structures sometimes callused and produced short hairy roots.

Table 4.6: The effect of plant growth regulators on bulblet regeneration and growth (after 52 weeks)

NAA : BA (mg ℓ^{-1})	Callus (%)	Abnormal organogenesis (%)	Shoots (%)	Bulblets (%)	Mean number bulblets / explant	Mean bulblet diameter (mm)
0 : 0	0	0	0	33	1.0[a]	6.0[a]
0 : 0.1	0	0	0	100	1.0[a]	7.0[a]
0 : 1	0	0	33	67	1.5[a]	6.3[a]
0.1 : 0	0	0	0	50	1.0[a]	11.5[a]
0.1 : 0.1	0	25	0	75	1.0[a]	9.5[a]
0.1 : 1	50	50	0	0	-	-
1 : 0	0	100	0	0	-	-
1: 0.1	50	50	0	0	-	-
1 : 1	0	100	0	0	-	-

Different letters show significant differences between treatments at the 5% level (ANOVA).

Similar trends were noted in the second experiment where a higher cytokinin to auxin ratio was used. Bulblets only developed in auxin-free media. An increase in the level of BA resulted in fewer shoots forming bulblets (Table 4.7). Bulblet development was noticeably absent in

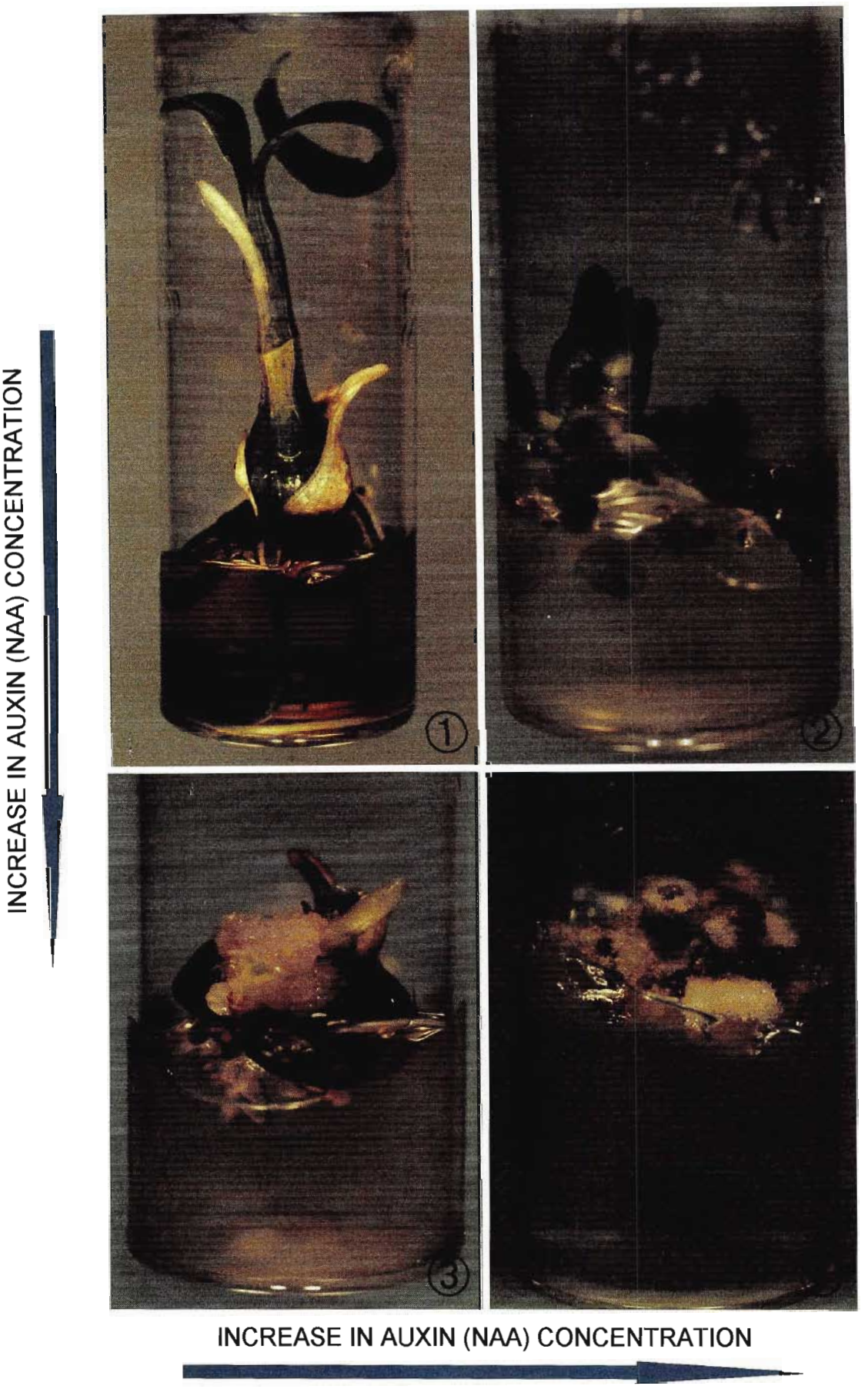


Figure 4.4: The effect of hormones on plantlet regeneration from twin-scales

1. Bulblet - 0 mg ℓ^{-1} NAA + 0.5 mg ℓ^{-1} BA
2. Shoots - 0 mg ℓ^{-1} NAA + 2.0 mg ℓ^{-1} BA
3. Callus - 0.5 mg ℓ^{-1} NAA + 1.0 mg ℓ^{-1} BA
4. Abnormal organogenesis - 1 mg ℓ^{-1} NAA + 1.0 mg ℓ^{-1} BA

those treatments that contained both auxin and cytokinin. Instead, the scales became swollen and vitrified, sometimes with a crystalline callus giving them a crustose appearance.

Table 4.7: The effect of plant growth regulators on bulblet regeneration and growth (after 23 weeks)

NAA : BA (mg ℓ^{-1})	Callus + Abnormal organogenesis (%)	Shoots (%)	Bulblets (%)	Mean number bulblets / explant	Mean bulblet diameter (mm)
0 : 0	-	38	13	1.0[a]	5.0[a]
0 : 0.5	-	11	89	1.3[a]	4.7[a]
0 : 1	-	83	22	1.0[a]	4.5[a]
0 : 2	-	90	0	-	-
0.5 : 0	42	0	8	1.0[a]	10.0[b]
0.5 : 1	88	-	-	-	-
0.5 : 2	80	-	-	-	-
1: 0	36	-	-	-	-
1 : 1	82	-	-	-	-
1 : 2	17	-	-	-	-

Different letters show significant differences between treatments at the 5% level (ANOVA).

Discussion

Spontaneous bulblet development in *C. moorei* twin-scales was observed in the absence of plant growth regulators but bulblets also formed when low concentrations of auxin and cytokinin were used. Explants of *Gladiolus* (STEINITZ & LILIEN-KIPNIS, 1989; DE BRUYN & FERREIRA, 1992), *Narcissus* (STEINITZ & YAHEL, 1982), *Nerine*, *Hippeastrum* (ALDERSON & RICE, 1986), *Lilium* (TAKAYAMA & MISAWA, 1979; MAESATO *et al.*, 1994) and *Vallota purpurea* (KUKULCZANKA & KROMER, 1988) respond equally well by producing storage organs even without hormones. In fact, more *Gladiolus* corms were produced on the control medium than on media containing hormones (DE BRUYN & FERREIRA, 1992).

Other geophytes have an absolute requirement for cytokinins. In potato, for example, cytokinins are a prerequisite for tuberization (PALMER & SMITH, 1969; ABBOTT & BELCHER, 1986; cited by DANTU & BHOWJANI, 1995) while in *Lilium* they are needed to stimulate differentiation in bulb scales (TAKAYAMA & MISAWA, 1980). Although cytokinins were not essential for bulblet formation in *C. moorei*, low levels of 0.1 - 0.5 mg ℓ^{-1} BA were shown to improve regeneration. *Nerine* hybrids have a similar requirement for BA at this

concentration (CUSTERS & BERGERVOET, 1992). Not only do cytokinins improve the regeneration potential of bulb explants, but also the numbers of bulblets produced. Using the twin-scale technique in *Hippeastrum*, a soak in 100 mg ℓ^{-1} BA or kinetin was found to increase the number of bulblets (HUANG *et al.*, 1990). The inclusion of cytokinin also yielded an increase in the number of bulblets formed per explant in *Vallota purpurea* (KUKULCZANKA & KROMER, 1988), while, multiple bulblet formation in *Narcissus* occurred at levels of 2 mgℓ^{-1} BA (HUSSEY, 1978) and in *Crinum* 'Ellen Bosanquet' at 35.5 µM (ULRICH et al., 1999). As many as twenty bulblets could be produced when BA was included at a slightly higher concentration (4 - 12 mg ℓ^{-1}) together with NAA (HUSSEY, 1978). Ginzburg and Ziv (1973) found that raising the level of kinetin resulted in larger cormels of *Gladiolus*, but that growth was superior when kinetin was used in combination with NAA. In contrast to the promotory effects of cytokinins on bulblet development, high levels may, in some instances, suppress their growth, as in the case of *Gladiolus* corms (STEINITZ & LILIEN-KIPNIS, 1989; DANTU & BHOJWANI, 1995) and tuberization in *Dioscorea* (FORSYTH & VAN STADEN, 1984;cited by DANTU & BHOJWANI, 1995). Cytokinins were also found to inhibit bulblet regeneration in *Narcissus* cultivars, either with or without the auxin NAA (CHOW *et al.*, 1992[a]). Ngugi *et al.* (1998) noted that more *Drimia robusta* bulblets formed with the highest concentration of BA but that these were abnormally formed. The inclusion of cytokinins may even lead to explant death e.g. *Hippeastrum hybridum* (MII *et al.*, 1974). Mii *et al.* (1974) postulate that this was because high endogenous levels made external applications ineffective. The requirement for cytokinin may be influenced by available sugar. Ginzburg and Ziv (1973) found that exogenous cytokinin was needed for corm formation in *Gladiolus,* which Dantu and Bhojwani (1995) attribute to the comparatively low level of sucrose present, namely 3%.

Bulblet regeneration is inhibited as a consequence of including auxin, on its own, in the medium. Jacobs *et al.* (1992) found that 0.2 mg ℓ^{-1} or more of 2,4-D resulted in a decrease in the regeneration of *Nerine bowdenii* bulblets. Increasing the levels of NAA beyond even 0.01 mg ℓ^{-1} led to the suppression of the regeneration response in *Lilium* (MAESATO *et al.*, 1994). Infrequent bud development was noted in *Hippeastrum hybridum*, also following the use of high concentrations of NAA. This was sometimes accompanied by abnormal root development (MII *et al.*, 1974). A similar response was reported by Huang *et al.* (1985; cited by OKUBO, 1993) to occur in twin-scale propagation. Although auxin reduced the number of bulblets per twin-scale in *Hippeastrum hybridum*, this was thought to be desirable since growers only want one bulblet per explant (FERNANDO *et al.*, 1994). In other bulbous crops, auxins may induce the development of *in vitro* bulblets e.g. *Nerine bowdenii* (PIERIK & IPPEL, 1977), *Narcissus* cultivars (CHOW *et al.*, 1992[a]) and *Vallota purpurea* (KUKULCZANKA & KROMER, 1988). Not all auxins produce the same effect. In *Vallota purpurea*, for example (KUKULCZANKA & KROMER, 1988), NAA resulted in an increase in the number of regenerating explants while the same effect was not observed using IAA. The

frequency with which this occurs is reported to be significantly higher in the presence of high (9%) sucrose concentrations (CHOW *et al.*, 1992[a]).

The inclusion of low levels of auxin in the medium does, however, result in an increase in bulblet diameter for *C. moorei*. In *Eucharis grandiflora*, bulblet weight was also found to improve if auxins were present (PIERIK *et al.*, 1983).

In the case of *C. moorei*, fewer bulblets developed at the highest concentrations of hormones used. Auxins and cytokinins are known to inhibit bulblet formation in bulbs like *Narcissus tazetta* (STEINITZ & YAHEL, 1982), *Nerine* (MOCHTAK, 1989) and *Gethyllis* (DREWES & VAN STADEN, 1994). They even decrease organogenesis when increased concentrations are used (DREWES & VAN STADEN, 1994). Maesato *et al.* (1994) also noted that the frequency of abnormal bulblets was higher in media containing significantly higher levels of plant growth regulators. Different malformations were observed such as increased leaf production and bulblets with abnormal roots or enlarged bulb-like structures at their base instead of roots.

The role of hormones in regulating storage organ formation has, in other experimental systems, been ascribed to their involvement in regulating assimilate flow. This is achieved by determining the main sink in the system (GINZBURG & ZIV, 1973). Cytokinins and gibberellins are particularly important in this respect (GINZBURG & ZIV, 1973). The fact that growth regulators are not required for bulblet induction is perhaps because endogenous levels are sufficiently high. Maesato *et al.* (1994) suggest that hormones involved in the morphogenic response are already present in the material such that regeneration occurs readily on hormone-free media. These are purported to be cytokinins or cytokinin-like compounds. If these are already present, the addition of low levels of auxins might establish an auxin-cytokinin balance required for organ regeneration. That would explain why the inclusion of small amounts of auxin and cytokinin were as effective in stimulating bulblet formation as the control. Wounding may also be responsible for this phenomenon.

CHARCOAL

Introduction

Charcoal has been used in media for propagating bulbs as it reduces browning and eliminates the decay of twin-scale bases (STEINITZ & YAHEL, 1982). This has been attributed to its ability to adsorp inhibitory compounds from the medium or those secreted by the explant (WEATHERHEAD *et al.*, 1978; HUGHES, 1981; PECK & CUMMING, 1986) thus rendering them ineffective (WANG & HUANG, 1976). It also improves bulblet regeneration, through direct organogenesis, and strongly influences the size of bulblets (STEINITZ & YAHEL, 1982; PECK & CUMMING, 1986). If excluded from the medium, bulblet induction and growth may be significantly reduced (STEINITZ & YAHEL, 1982). This may be due, in part, to the simulation of natural soil conditions (PROSKAUER & BERMAN, 1970; cited by WANG & HUANG, 1976).

Materials and methods

Charcoal was added to the medium at a concentration of 5 g ℓ^{-1}. Twin-scales were inoculated onto charcoal-free and charcoal-supplemented media and placed in the light (70.7 $\mu mol.m^{-2}.s^{-1}$; 16h:8h light:dark) at 25°C.

Results

Bulblet formation was greater in explants grown on media with charcoal than in twin-scales "deprived" of charcoal (Table 4.8). On average, each twin-scale produced 1.2 bulblets on media supplied with charcoal. These bulblets also had a greater average diameter (7.8 mm) and fresh weight (1.82 g) compared to those grown on charcoal-free media although a *t*-test did not show any significant differences (Figure 4.5). In general, there was a decrease in anthocyanin production and necrosis of twin-scales grown on media supplied with charcoal.

Table 4.8: The effect of charcoal on bulblet regeneration and growth (after 33 weeks)

Charcoal concentration (g ℓ^{-1})	Shoots (%)	Bulblets (%)	Mean number shoots / explant	Mean number bulblets / explant	Mean bulblet diameter (mm)	Mean bulblet fresh weight (g)
0	16	58	1.4[a]	1.1[a]	6.8[a]	0.82[a]
5	30	63	1.4[a]	1.2[a]	7.8[a]	1.82[a]

Different letters show significant differences between treatments at the 5% level (*t* - TEST).

Figure 4.5: The effect of charcoal on bulblet size
1. Bulblet - no charcoal
2. Bulblet - 5 g ℓ^{-1} activated charcoal

Discussion

The highest bulbing response was recorded for twin-scale explants that were grown on media containing charcoal. There were also a greater number of bulblets per explant and bulblets with a larger average diameter and higher fresh weight. Stimulatory effects of charcoal have been observed in *Paphiopedilum* (WANG & HUANG, 1976), lily (TAKAYAMA & MISAWA, 1980), *Eucrosia* (ZIV & LILIEN-KIPNIS, 2000) and *Narcissus* (LANGENS-GERRITS & NASHIMOTO, 1997; ZIV & LILIEN-KIPNIS, 2000). In *Narcissus tazetta* (STEINITZ & YAHEL, 1982), charcoal doubled regeneration and significantly influenced the size of the bulblets. There are a number of possible reasons for this. Firstly, charcoal is known to adsorb inhibitory compounds; either those in the medium or those secreted by the explant (WEATHERHEAD *et al.*, 1978; PECK & CUMMING, 1986). HMF (a mild growth inhibitor that is produced by sucrose degradation during autoclaving), for example, is bound by charcoal (WEATHERHEAD *et al.*, 1978). Secondly, hormones such as IAA, 2iP, NAA, kinetin and BA (HUGHES, 1981) are rendered inactive by charcoal (WEATHERHEAD *et al.*, 1978). By removing compounds that are responsible for unorganized growth or those that inhibit embryogenesis and root formation and elongation, charcoal may stimulate embryogenesis (FRIDBORG & ERIKSSON, 1975; HUGHES, 1981) and rooting (FRIDBORG & ERIKSSON, 1975; YEOMAN, 1986). One advantage of inducing direct organogenesis instead of callus on bulb scales is that the potential for genetic abnormalities is eliminated (PECK & CUMMING, 1986). It also reduces the number of culture steps and, therefore, decreases production time for marketable products like bulbs (PECK & CUMMING, 1986). The presence of charcoal was also found to reverse the inhibitory effects of BA (TAKAYAMA & MISAWA, 1980) and BA and NAA in combination (STEINITZ & YAHEL, 1982), on bulbil development from lily bulb scales. According to Takayama and Misawa (1980), the ability of activated charcoal to alter the internal physiological processes was presumably due to its regulation of cytokinin activity or its adsorption of substances likely to antagonise the cytokinin activity. The adsorption of BA (LESHAM, 1974; cited by TAKAYAMA & MISAWA, 1980), which is known to stimulate shoot production in *C. moorei* twin-scales at the expense of bulblet formation, may therefore account for the higher rate of bulblet regeneration observed in cultures grown on charcoal. Thirdly, charcoal is able to prevent browning and the decay of twin-scale bases in geophytes that produce exudates (STEINITZ & YAHEL, 1982; YEOMAN, 1986; ZIV & LILIEN-KIPNIS, 2000). As it darkens the medium (HUGHES, 1981), workers can approximate soil conditions (WEATHERHEAD *et al.*, 1978) such as those normally experienced by geophytic organs.

LIGHT

Introduction

Light also affects bulblet growth. Jacobs *et al.* (1992) reported both an increase in the number of bulblets produced per explant and an increase in bulblet fresh weight for *Nerine bowdenii* twin-scales grown in the light. The growth of *Lilium longiflorum* (LESHEM *et al.*, 1982) and *Narcissus tazetta* (STEINITZ & YAHEL, 1982) bulbs, however, was reduced under the same conditions. The repressive effect of light (STEINITZ & YAHEL, 1982) can be overcome by growing the explants in the dark as darkness stimulates bulblet formation in many bulbous species (VAN AARTRIJK & VAN DER LINDE, 1986).

Materials and methods

To determine the effect of light on shoot induction from twin-scales and on bulblet development, twin-scales were excised from flowering-size bulbs and placed on an M&S basal medium. Twin-scales were placed in the dark and light at 25°C. Light was provided at 70.7 $\mu mol.m^{-2}.s^{-1}$, in a cycle of 16 hours light and 8 hours dark.

To determine the effect of charcoal and light on bulblet regeneration, explants were inoculated on charcoal-free or charcoal-supplemented media (at 5 g ℓ^{-1}) and placed either in the dark or in the light.

Results

Light affected shoot initiation from twin-scales. A higher percentage of explants (79%) developed shoots when they were grown in the dark rather than the light (67%). The dark-grown twin-scales also produced significantly more shoots with a higher mean length compared to those exposed to light (Table 4.9).

Table 4.9: The effect of light on the induction of shoots from twin-scales (after 7 weeks)

Light	Shoots (%)	Mean number shoots / explant	Mean shoot length (mm)
Light	67	1.3^a	5.8^a
Dark	79	1.7^b	11.3^b

Different letters show significant differences between treatments at the 5% level (*t* - TEST).

While bulblet production occurred in both the light and dark, more explants developed bulblets when grown in the light (Table 4.10). The average bulblet diameter was also greater in the light (8.2 mm) compared to those in the dark (6.3 mm) but not significantly so (Figure 4.6).

Table 4.10: The effect of light on bulblet regeneration and growth (after 33 weeks)

Light	Shoots (%)	Bulblets (%)	Mean number shoots / explant	Mean number bulblets / explant	Mean bulblet diameter (mm)
Light	12	69	2.0	1.2[a]	8.2[a]
Dark	31	53	1.2	1.2[a]	6.3[b]

Different letters show significant differences between treatments at the 5% level (*t* - TEST).

The results of the combined effects of charcoal and light are tabulated below. The highest bulbing response was recorded for twin-scales grown on charcoal and in the light. These bulblets were larger than either those grown in the absence of charcoal or in the dark (Table 4.11).

Table 4.11: The effects of charcoal and light on bulblet regeneration and growth (after 33 weeks)

Charcoal concentration (g ℓ^{-1}) & Light	Shoots (%)	Bulblets (%)	Mean number shoots / explant	Mean number bulblets / explant	Mean bulblet diameter (mm)
0 : Light	13	60	2.0[a]	1.1[a]	7.8[ab]
0 : Dark	19	56	1.0[a]	1.1[a]	5.7[b]
5 : Light	9	82	2.0[a]	1.2[a]	8.6[a]
5 : Dark	44	50	1.3[a]	1.3[a]	6.9[ab]

Different letters show significant differences between treatments at the 5% level (ANOVA).

Discussion

Shoot induction in *C. moorei* twin-scales took place in the dark. The induction of organogenesis in explants first placed in the dark has been noted for members of the Iridaceae and Liliaceae and in *Hippeastrum hybridum*, *Nerine bowdenii* and also *Iris hollandica* (VAN DER LINDE *et al.*, 1988). Placing explants in the dark is standard practice for the culture of *Narcissus confusus* from twin-scales (BERGOÑÓN *et al.*, 1996). The reason for this, as Hughes (1981) suggests, may be related to starch accumulation.

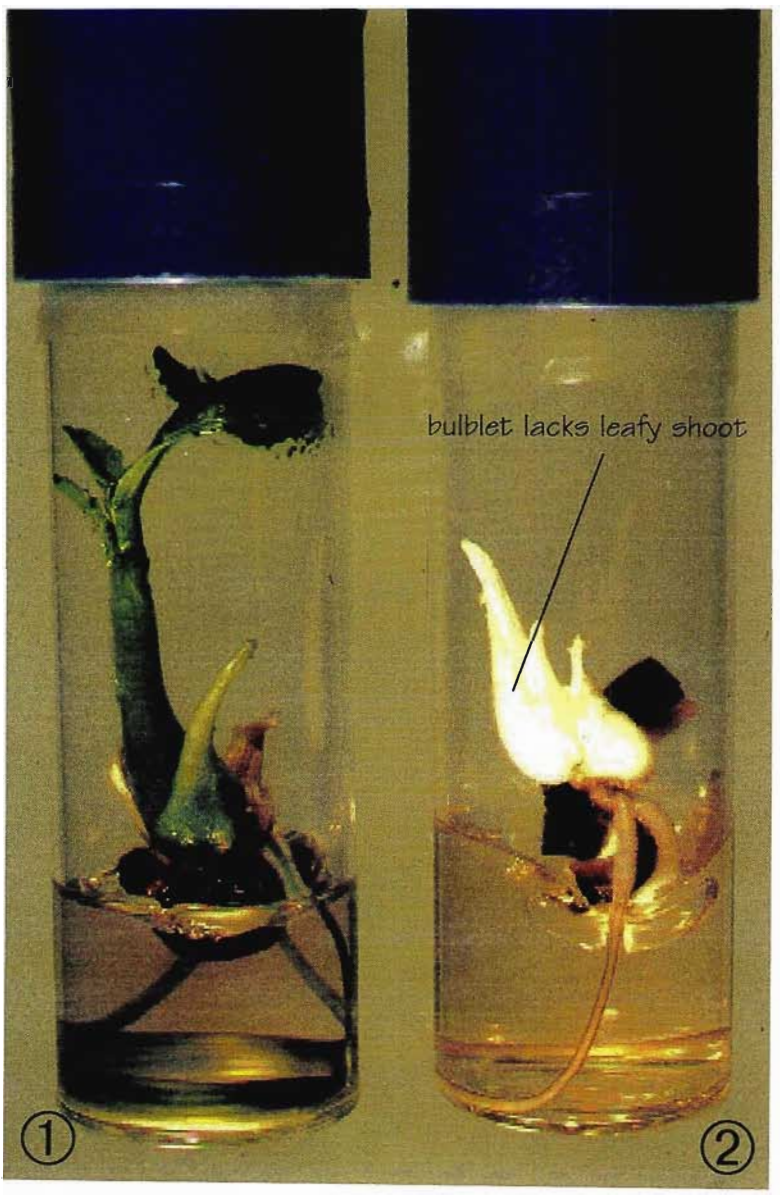


Figure 4.6: The effect of light on bulblet size
1. Bulblet grown in light
2. Bulblet grown in dark

While light was not a requirement for the induction of shoots from *C. moorei* twin-scales, it was essential for the normal development of bulblets. Darkness did not enhance bulblet development in *Crinum* 'Ellen Bosanquet' (ULRICH *et al.*, 1999). Similarly, growing *Allium sativum* (ZEL *et al.*, 1997) and *Lilium japonicum* (MAESATO *et al.*, 1994) explants in the dark had no stimulatory effect on bulb formation. Not only did light enhance the number of bulb scales per bulblet but it was also necessary for leaf emergence and subsequent growth (MAESATO *et al.*, 1994). Light may also result in an increase in the number of bulblets per explant and in their fresh weight (JACOBS *et al.*, 1992). A low gamma-irradiation dose stimulated bulblet development in *Lilium rhodopaeum* (STANILOVA *et al.*, 1994). This is contrary to reports of light reducing or inhibiting regeneration and growth of bulbs and corms (LESHEM *et al.*, 1982; STEINITZ & YAHEL, 1982; DANTU & BHOJWANI, 1995) and, of darkness promoting bulblet development (PIERIK & IPPEL, 1977; BACKHAUS *et al.*, 1992; JACOBS *et al.*, 1992; LANGENS-GERRITS & NASHIMOTO, 1997). In some instances darkness may also promote rooting and leaf development (PIERIK & IPPEL, 1977). In *Nerine bowdenii* it increased the number of explants forming bulblets (JACOBS *et al.*, 1992). Micropropagated bulblets of *Lilium* species are conventionally grown in the dark (MARINANGELI & CURVETTO, 1997) because, *Lilium longifolium*, for example, produces larger bulblets. Potato tubers form in the dark *in vivo*. Tuberization *in vitro* can also occur in promoting medium in the dark (ABBOTT & BELCHER, 1986). *In vitro* corm formation, however, is similar in both the light and dark for *Gladiolus* species (STEINITZ *et al.*, 1991; cited by DANTU & BHOJWANI, 1995) and in *Hyacinth* cultivars, regeneration and bulb weight are identical in both continuous light and darkness (PIERIK & RUIBING, 1973).

The promotory effects of light and charcoal were thus enhanced when twin-scales of *Crinum moorei* were grown on charcoal-supplemented media and in the light.

TEMPERATURE

Introduction

Temperature requirements for different species may differ, for although most tissues are cultured at 25°C, the optimum temperature for bulbous species is sometimes lower (YEOMAN, 1986). Higher culture temperatures of 15 - 30°C are known to stimulate the bulbing of shoots (VAN AARTRIJK & VAN DER LINDE, 1986).

Materials and methods

Twin-scales were placed in growth chambers and incubated at 15°C, 20°C, 25°C and 30°C, with a 16:8 light regime and light intensity of 70.7 $\mu mol.m^{-2}.s^{-1}$.

Results

Of the twin-scales incubated at 30°C, 78% produced shoots. While fewer explants produced shoots at temperatures below 30°C (Table 4.12) some showed multiple shoot production. The numbers of shoots produced at 15°C and 20°C were significantly different to those grown at higher temperatures. All the shoots grown at 30°C lacked chlorophyll and appeared vitrified (Figure 4.7). Lower temperatures of 15°C appeared to stunt shoot growth. The best bulbing response was achieved at a temperature of 25°C. Here, 63% of the explants developed bulblets with an average diameter of 8.4 mm.

Table 4.12: The effect of temperature on bulblet regeneration and growth (after 22 weeks)

Temperature (°C)	Shoots (%)	Bulblets (%)	Mean number shoots / explant	Mean number bulblets / explant	Mean bulblet diameter (mm)
15°C	53	0	4.2[a]	-	-
20°C	44	56	6.2[a]	1.0[a]	8.3[a]
25°C	13	63	1.0[b]	1.0[a]	8.4[a]
30°C	78	0	1.0[b]	-	-

Different letters show significant differences between treatments at the 5% level (ANOVA).

Discussion

Temperatures of 20 and 25°C stimulated bulblet development since no bulblets were observed in explants grown at 15°C or at 30°C. This appears to be a normal response as most tissues are cultured at 25°C (YEOMAN, 1986), including bulbs like *Nerine sarniensis* (VISHNEVETSKY *et al.*, 1997), *Narcissus tazetta* (STEINITZ & YAHEL, 1982) and *Narcissus* cultivar "Lord Nelson" (SEABROOK & CUMMING, 1982). In the latter case, the largest number of bulbs were formed at 25°C compared to temperatures of 15, 20 and 30°C. A constant temperature of 25°C was also conducive to shoot production which was greatest at this temperature (SEABROOK & CUMMING, 1982). Bulblet regeneration and weight in *Eucharis grandiflora* were promoted at temperatures of 21 - 25°C (PIERIK *et al.*, 1983) as was the stimulation of bulblet development in *Nerine* species (PIERIK & IPPEL, 1977).

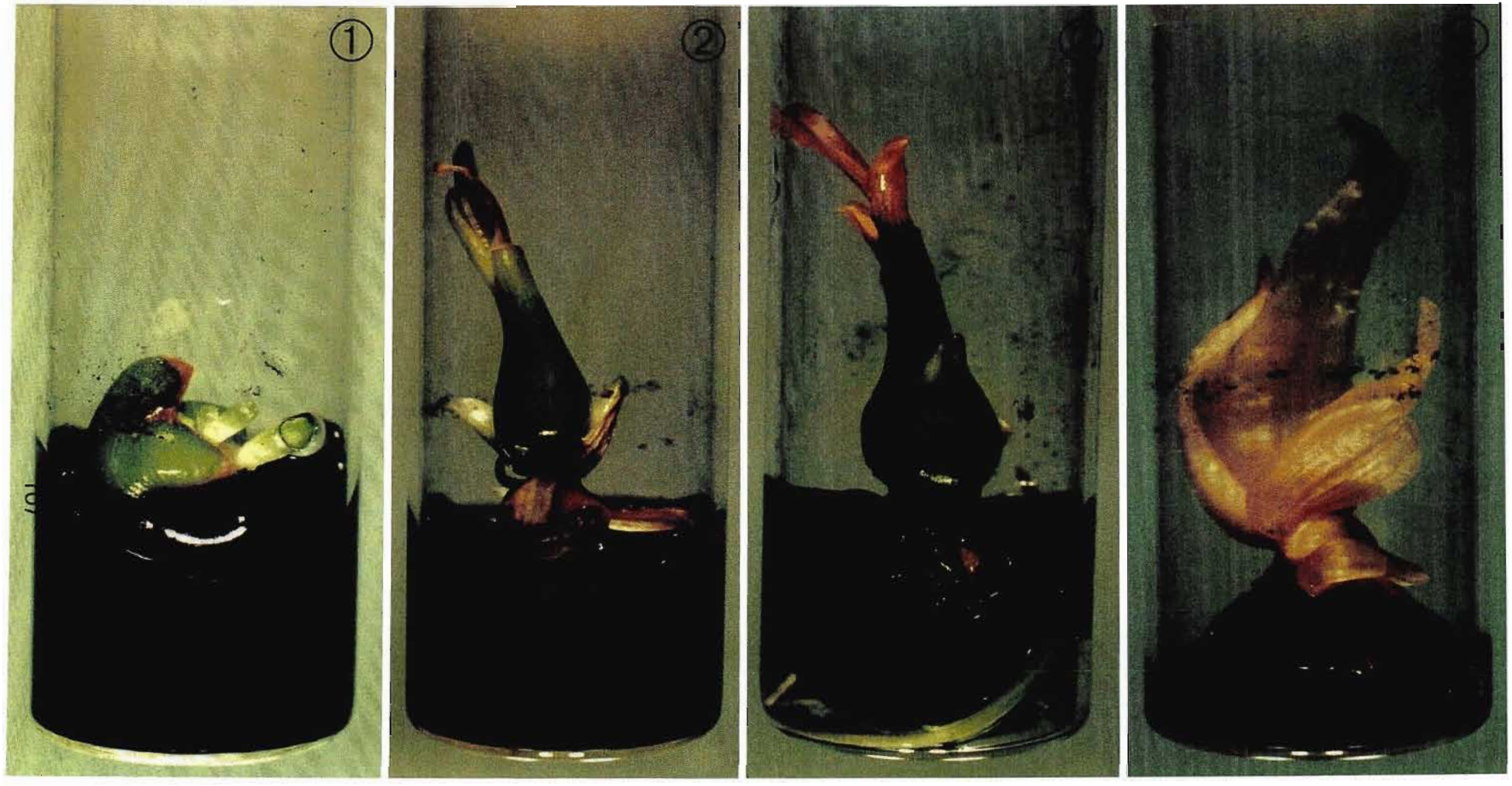


Figure 4.7: The effect of temperature on bulblet regeneration and growth

1. Stunted shoot - 15°C
2. Bulblet - 20°C
3. Bulblet - 25°C
4. Abnormal shoot - 30°C

Bulblet growth in *Nerine bowdenii* (JACOBS *et al.*, 1992) was optimal in the temperature range 17 - 22°C. At higher temperatures the percentage explants forming bulblets was noticeably lower. The speed at which hyacinth scales regenerated could be increased by increasing the temperature of incubation. Although the number of bulblets formed per explant was optimal at 13°C, bulblet weight was higher at 21.6°C or at 24.8°C (PIERIK & RUIBING, 1973).

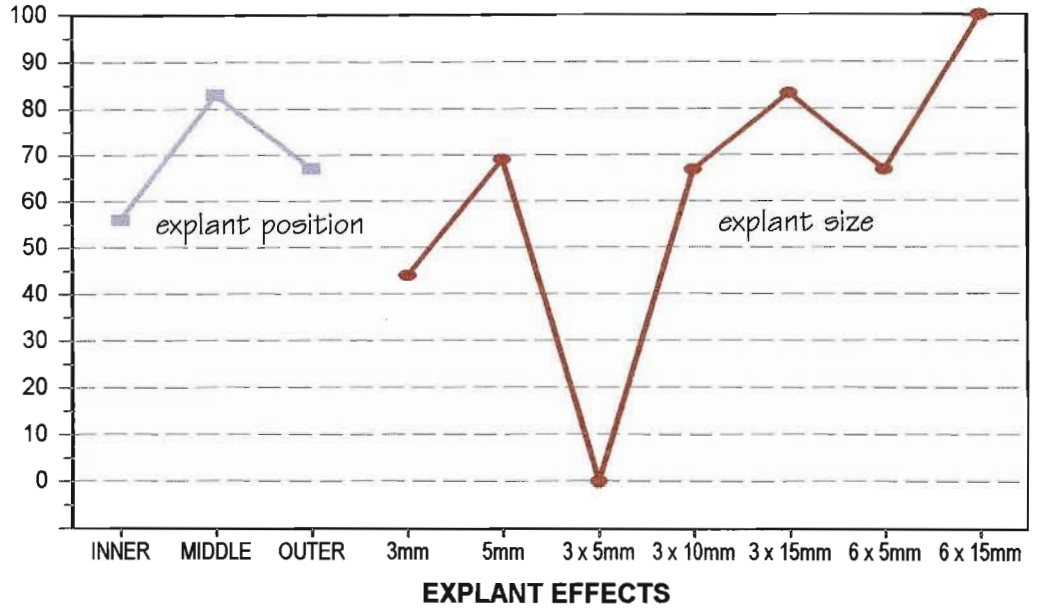
In other bulbous species cold temperatures are required to induce swelling at the base of the shoots where meristematic centres occur (TAEB & ALDERSON, 1990). In the case of *Iris hollandica*, optimal regeneration occurred only at temperatures below 25°C (VAN DER LINDE *et al.*, 1988) while 20°C was better than 25°C for greater bulblet production in *Lilium japonicum* (MAESATO *et al.*, 1994). Lower temperatures of 15°C or 18°C are often used to stimulate bulblet development in *Galanthus* (YEOMAN, 1986), *Gladiolus tristis* (DE BRUYN & FERREIRA, 1992) and *Narcissus* cultivars (YEOMAN, 1986). Tulips are subjected to a cold treatment of 4°C or 5°C (VAN DER LINDE *et al.*, 1988) for 12 weeks to bring on bulbing. In this instance, the low temperature requirement may be associated with sucrose accumulation which, at this temperature, is at high levels in the shoots (TAEB & ALDERSON, 1990). High sucrose levels are known to induce dormancy by inhibiting shoot formation (TAKAYAMA & MISAWA, 1980). Hanks *et al.* (1986) noted that starch hydrolysis was enhanced at 15°C, and that strong bulbil production from twin-scales followed storage at this temperature. Bulbil production is known to be inhibited in some cold stored bulbs. Endogenous levels of GA increase in these conditions which might explain why bulbil production is poor (HANKS *et al.*, 1986).

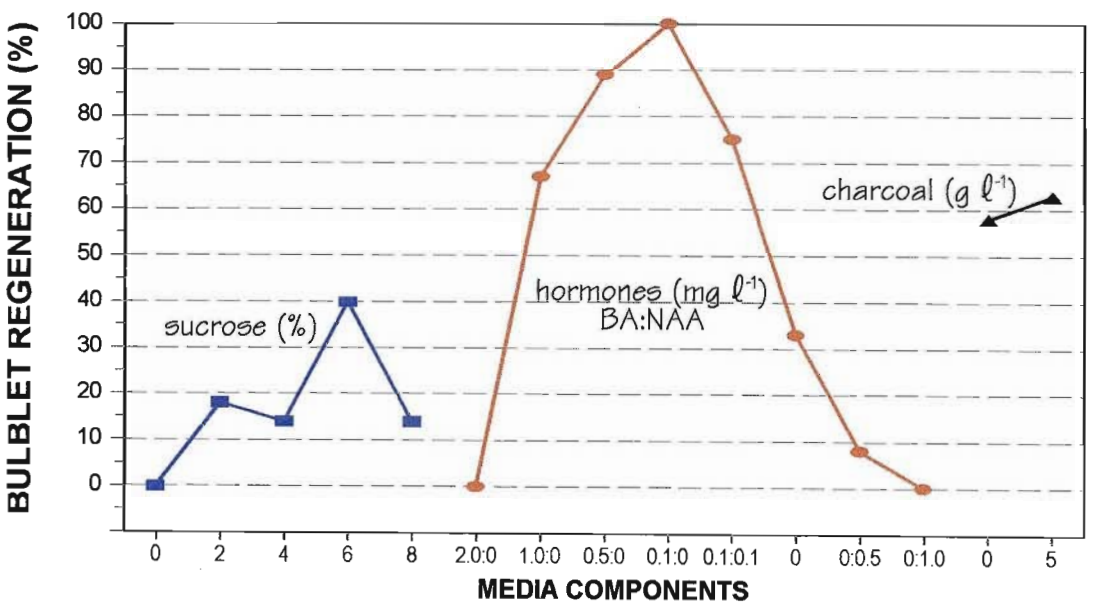
Higher temperatures are needed to initiate bulbing of *C. moorei* perhaps because they approximate the conditions in the bulb's native habitat. Murashige (1974) alluded to this in suggesting that *in vitro* studies should focus on the temperature requirements of temperate and desert-region plants. However, temperatures of 30°C and more inhibited bulb formation. This may reflect the poor availability or conservation of soluble sugars at this temperature. Yamagishi (1998) found that the suppression of bulblet enlargement at high temperatures was accompanied by reduced sugar uptake from the medium. The cause of this may have been poor root development, or the induction of dormancy at high temperatures (YAMAGISHI, 1998). Furthermore, accumulated carbon compounds were increasingly used during respiration at these higher temperatures. Seabrook and Cumming (1982) came to the conclusion that "temperature effects on plant tissues may closely resemble enzyme activity curves for temperature".

CONCLUSIONS

Those factors that are responsible for improving bulblet regeneration include: explant position, notably those from position two; explants with a diameter and length greater than 3mm; a sucrose concentration of 6%; low (0.1 mg ℓ^{-1}) levels of the cytokinin BA; 5 g ℓ^{-1} activated charcoal; light and a temperature of 25°C. (See Figure 4.8).

Twin-scales rarely produced more than two bulblets per explant. Although the use of plant growth regulators could be expected to improve bulblet yield, it may be that plantlets formed only from existing meristems, which would be limited by the size of the twin-scale. Kukulczanka and Kromer (1988) suggest that the origin of bulblets, namely, at the base of the scale, confirms this phenomenon and that apical dominance also strongly influences the number of growing explants. Bulblets were, therefore, used as a source of secondary explants to improve rates of multiplication, as outlined below.





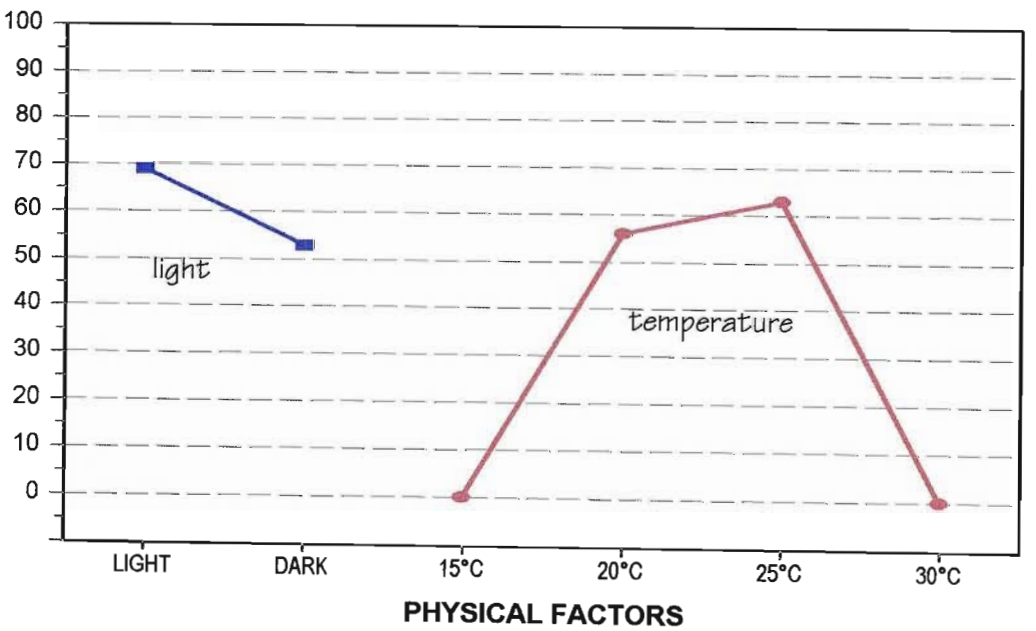


Figure 4.8: A summary of factors affecting bulblet regeneration and growth

Multiplication of the propagules

INTRODUCTION

The multiplication of propagules is most often via one of two pathways for scaled-up micropropagation; either division of (1) shoot clumps or (2) culture-derived bulblets.

Some workers advocate the use of shoot clumps in multiplying bulbous crops. However, this may lead to a decrease in the propagation rate with each cycle and poor growth after transfer to the soil (LANGENS-GERRITS *et al.*, 1997) unless bulblets are allowed to develop in each cycle.

Further multiplication of bulblets can be achieved by cutting newly formed bulblets into halves (HUSSEY, 1982; SQUIRES & LANGTON, 1990; CUSTERS & BERGERVOET, 1992; SLABBERT *et al.,* 1993); by "decapitating" them to remove either the upper third (CUSTERS & BERGERVOET, 1992) or the upper half - leaving 1 - 3 mm - (LEFFRING, 1983; cited by CUSTERS & BERGERVOET, 1992) or quartering bulblets when a bulb size of at least 0.8 - 1.2 cm is reached (PIERIK *et al.*, 1990). The removal of the upper half of the bulb reduces growth of the main shoot (HUSSEY, 1986) which results in the development of secondary adventitious bulblets (YEOMAN, 1986). This may only be possible if the bulblets are more than 100 mg in weight as no meristematic cells are present in the axils of very young bulblets (GROOTAARTS *et al.*, 1981; cited by CUSTERS & BERGERVOET, 1992). The addition of BA to the medium caused a dramatic increase in the number of new plantlets in subcultured bulblets of *Nerine* hybrids (CUSTERS & BERGERVOET, 1992). Multiplication rates could, therefore, be increased by removing the upper half of the bulblets and adding BA to the medium. These methods possibly reduce dormancy problems commonly encountered in the micropropagation of bulbous ornamentals (GEORGE & SHERRINGTON, 1984; cited by CUSTERS & BERGERVOET, 1992) although regeneration is reported to decline with every new subculture (STEINITZ & YAHEL, 1982).

MATERIALS AND METHODS

To achieve further bulblet formation and to improve the rates of multiplication, *in vitro* grown bulblets (3 - 5 mm) were either cut in half through the basal plate or cut into quarters. In other cases, the upper half or two thirds of the bulblet were removed (Figure 4.9). Leaves and roots were removed before the explants were inoculated onto the M&S medium which had not been supplied with hormones.

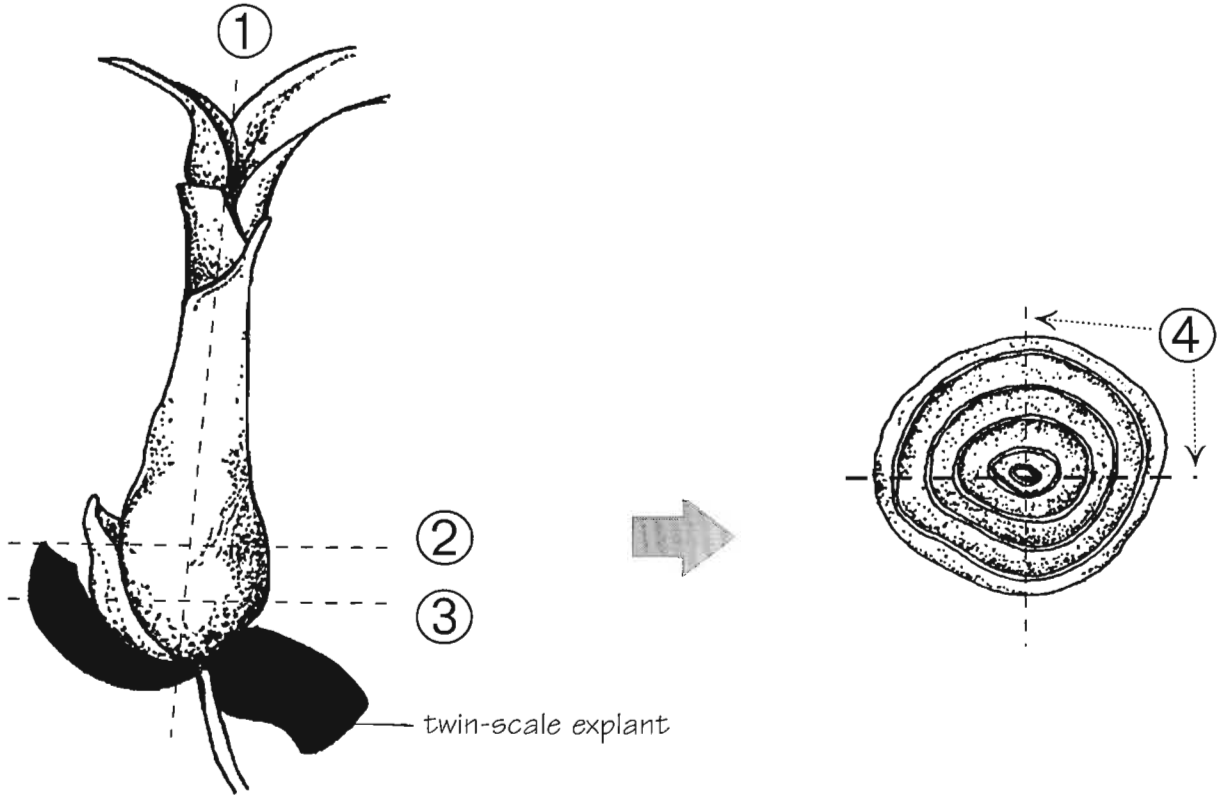


Figure 4.9: Methods of trimming *in vitro* bulblets for subculture

1. Bulblet cut vertically in half
2. Upper half of bulblet removed
3. Upper two-thirds of bulblet removed
4. Bulblet cut into quarters

In vitro grown bulblets that had been cut in half (i.e. vertical median cut) or trimmed to one third of their size were placed on M&S media supplemented with 0, 0.1, 1 or 10 mg ℓ^{-1} BA. In order to determine the effect of light on shoot regeneration from cut bulblets, bulblets were grown in the light (70.7 $\mu mol.m^{-2}.s^{-1}$; 16h:8h light:dark regime) or in continuous darkness.

RESULTS

After two weeks in culture the central bulb scales of "decapitated" bulblets elongated into leafy shoots. Leafy shoots also developed in "half" bulblets. However, these showed strong curvature toward their adaxial surface and often penetrated the culture medium. Roots developed from the basal plate in both treatments. Bulblets which had been cut in half, each formed a single new bulblet. After nine months in culture these had attained the size of approximately 5 - 8 mm in diameter. Bulblets that had been cut into quarters turned black and did not produce shoots.

The highest percentage of newly formed shoots was observed at the highest concentration of BA tested, namely 10 mg ℓ^{-1} (Table 4.13). This occurred both in the "halved" bulblets and those that had been cut to one third of their original size (Figure 4.10). All the explants that responded developed multiple shoots, with an average of 7.3 and 7.9 shoots per explant. Sometimes these developed alongside nodular callus (Figure 4.10). As the concentration of BA increased from 0 to 10 mg ℓ^{-1}, there was a decline in the number of bulblets produced (Figure 4.11). Most bulblets developed where BA levels were lowest i.e. 0.1 mg ℓ^{-1}.

Table 4.13: The effect of the hormone BA on the production of propagules from *in vitro* bulblets (afer 27 weeks)

BA (mg ℓ^{-1})	"½" Bulblets			"⅓" Bulblets		
	Shoots (%)	Mean number of shoots / explant	Bulblets (%)	Shoots (%)	Mean number of shoots / explant	Bulblets (%)
0	0	-	13	0	-	0
0.1	33	1.0[a]	33	40	4.0[a]	40
1	60	5.3[a]	20	22	5.0[a]	0
10	75	7.3[a]	0	88	7.9[a]	0

Different letters show significant differences between treatments at the 5% level (ANOVA).

Bulblets that had been divided developed shoots in both the light and dark. The "half" bulblets performed better than those that had been trimmed close to the basal plate with 60% of the bulblets producing shoots following this method of trimming. Bulblets developed only in the

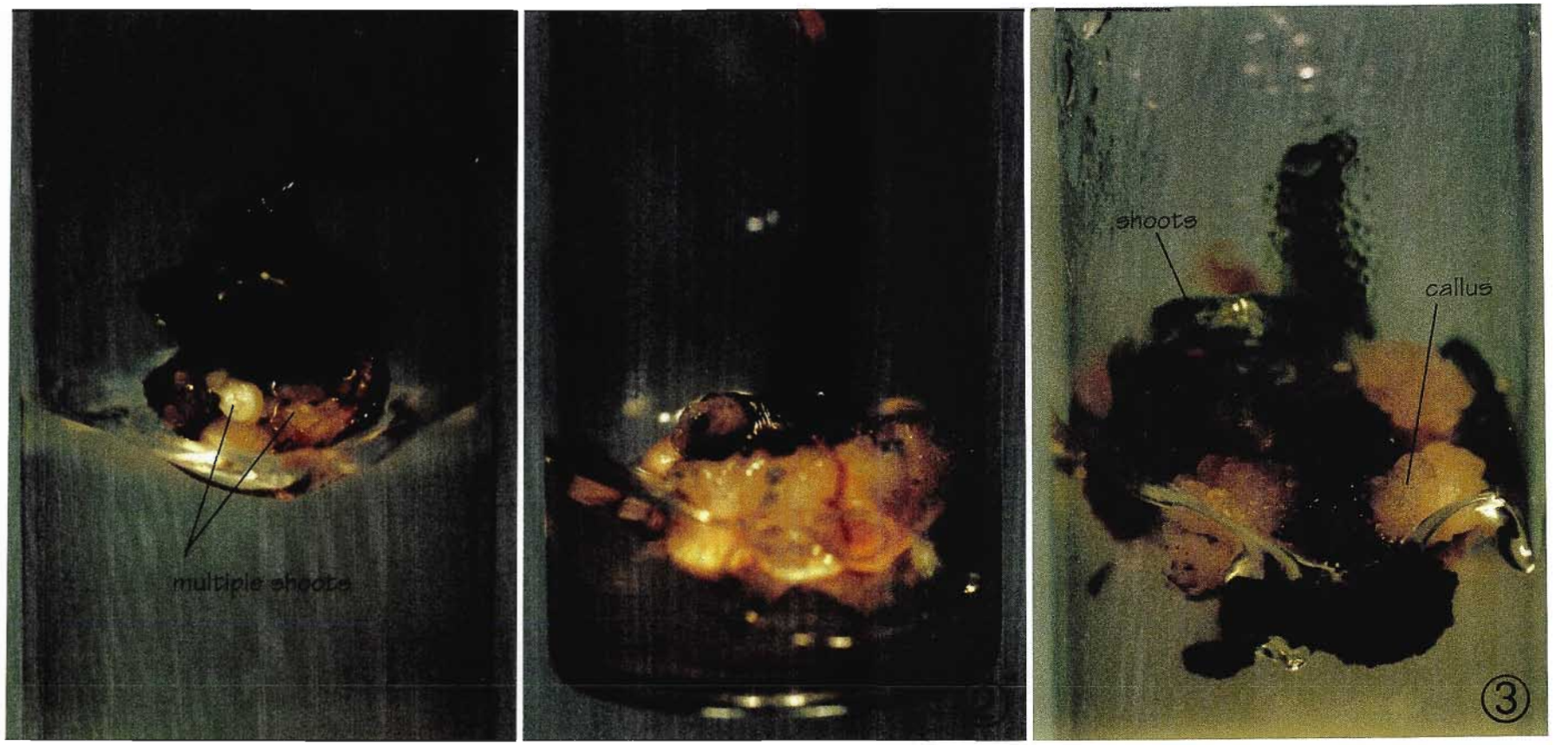


Figure 4.10: Secondary shoots from *in vitro* grown bulblets

1. Shoots in the axis of adjacent bulb scales of "half" bulblets
2. Shoots arising between scales of bulblets trimmed close to the basal plate ("⅓" bulblets)
3. Shoots and callus initiated on hormone-supplemented media

Figure 4.11:The effect of the hormone BA on multiple shoot production from *in vitro* bulblets

light (no bulblets were observed in cultures that were grown in continuous darkness). More (57%) of the "half" bulblets produced new bulblets compared to those that had their upper two thirds removed (Table 4.14). The bulblets were also larger. Overall, the response of the halved bulblets was superior with 51% of the bulblets producing new shoots or bulblets (Figures 4.12 and 4.13).

Table 4.14: The effect of light on the production of propagules from *in vitro* bulblets (after 27 weeks)

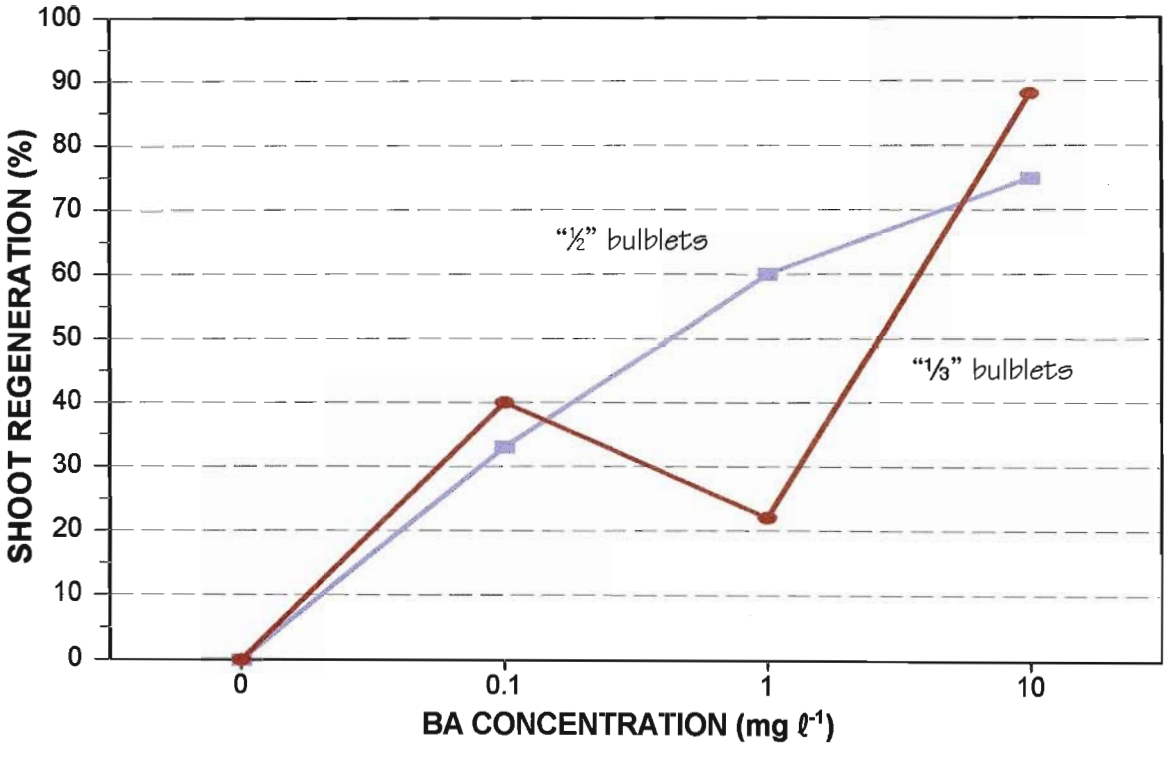
Light	"½" Bulblets			"⅓" Bulblets		
	Shoots (%)	Mean number of shoots / explant	Bulblets (%)	Shoots (%)	Mean number of shoots / explant	Bulblets (%)
Light	0	-	57	25	1.0	25
Dark	60	1.0	0	25	1.0	0

DISCUSSION

In general, the growth of subcultured bulblets was slow. However, when one considers that many twin-scales can be dissected from a mature bulb and that these readily form new bulblets which can be used for further multiplication, regeneration rates are comparable to those of other bulbs e.g. *Amaryllis belladonna* (DE BRUYN *et al.*, 1992) and *Hippeastrum* (HUANG *et al.*,1990).

The most successful method of bulblet multiplication was to cut the bulblets longitudinally in half. New plantlets developed between the scales in both the "half" bulblets and those that had their upper parts removed. Bulblets of *C. macowanii* which are at least 5 mm in diameter are also trimmed and split into halves (SLABBERT *et al.*, 1993). *Narcissus tazetta* produces bulblets by splitting culture-derived bulblets into halves (STEINITZ & YAHEL, 1982) as do some other *Narcissus* (HUSSEY, 1982[b]) and *Nerine* species (PIERIK *et al.*, 1981; cited by CUSTERS & BERGERVOET, 1992; SQUIRES & LANGTON, 1990). In *Nerine* hybrids, new plantlets formed more frequently in tissue cultured bulblets from which the upper half had been removed compared to those where only the upper third was cut away (CUSTERS & BERGERVOET, 1992). Axillary and adventitious shoots proliferate freely in *Iris*, *Lilium* and some *Tulipa* hybrids even when *in vitro* shoots are trimmed to within 2 - 3 mm of the basal plate (HUSSEY, 1982[a]). Shoot clumps of *Narcissus* cultivars are routinely subcultured by cutting down to the basal plate and removing all green tissue. This method of "severe cutting"

Figure 4.12: A comparison of bulblet growth between ① "½" bulblets and ② "⅓" bulblets



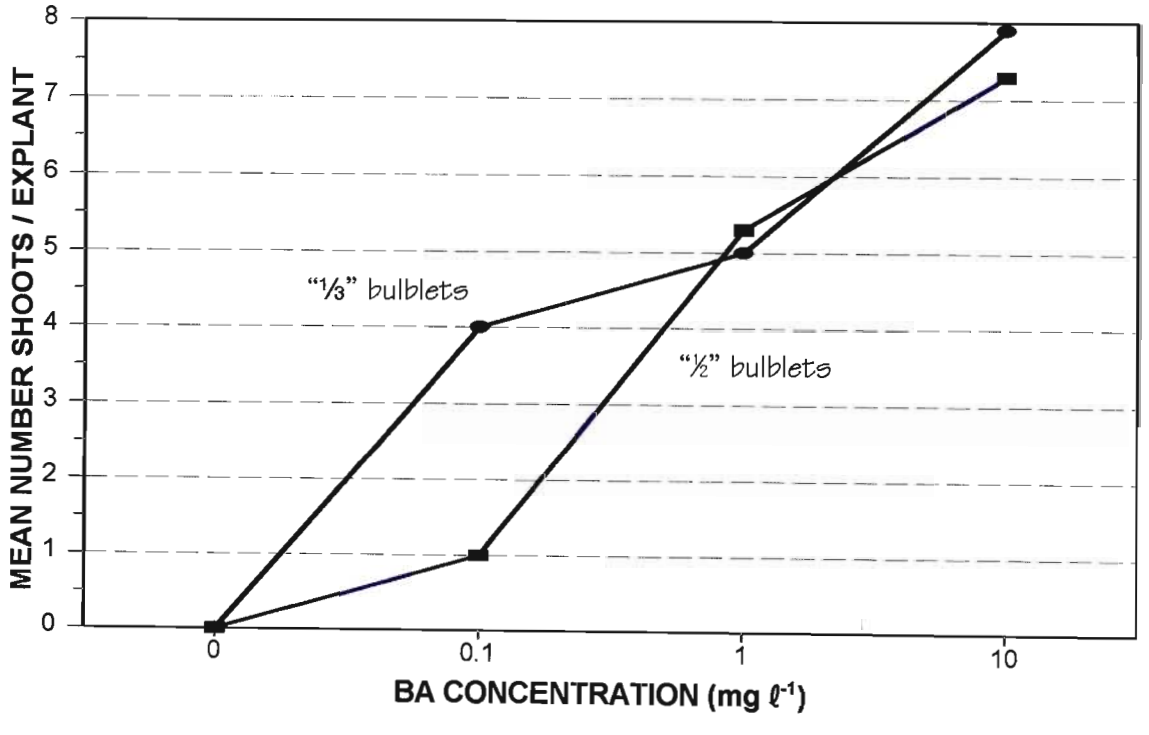


Figure 4.13: The effect of increasing the BA concentration on shoot regeneration from trimmed bulblets

doubled multiplication while no leaf multiplication occurred in cultures which were subject to "normal cutting" (CHOW *et al.*, 1992[b]). A tenfold multiplication was observed in the cultivar Lord Nelson as described by Seabrook (1990; cited by CHOW *et al.*, 1992[b]). It therefore appears that the severity of trimming and, in particular, the destruction of the shoot apex, affect axillary shooting. Species characterized by strong apical dominance e.g. *Allium*, *Hyacinth*, *Nerine* (HUSSEY, 1982[a]) and *Narcissus* (HUSSEY, 1986), receive a wedge-shaped cut in the basal plate in order to destroy the main apex (HUSSEY, 1982[a]). Sometimes two shallow cuts are made at right angles over the apex (HUSSEY, 1986). Dormant *Freesia* bulblets resume growth if the crowns are cut out and they are split into two (HUSSEY, 1976). According to Flint (1986; cited by CHOW *et al.*, 1992[b]), apical dominance is exerted by the basal plate in *Narcissus*. Thus, any technique that cuts down to or even into the basal plate of bulbs could be used to break dominance and stimulate shoot production. The advantage is that loss of vigour, caused by the onset of dormancy and senescence, is either prevented or delayed (HUSSEY, 1986; CHOW *et al.*, 1992[b]). Another factor which appears to affect the response of split *in vitro* shoots, is the vigour of the original shoot before splitting. Hussey (1982[b]) reported that vigorous shoots of *Narcissus* yielded more secondary shoots than those that grew more slowly. They were also more sensitive to the level of hormones in the medium. The report that no meristematic cells are present in the axils of very young bulblets (GROOTAARTS *et al.*, 1981) may explain why smaller explants from cultured bulblets of *Crinum moorei* did not produce shoots. Pierik and Ippel (1977) also concluded that the small number of bulblets that formed on bulblets (that had been split in half) was due to a lack of sufficient scale and basal plate tissue. Larger bulblets (75 - 350 mg) with more scales are therefore used, with the added advantage that plants are produced more quickly (CUSTERS & BERGERVOET, 1992). *In vitro* grown bulblets of *Amaryllis belladonna*, however, proved the most regenerative explants when divided into four parts (DE BRUYN *et al.*, 1992). Each quarter developed at least one bulblet. Hussey (1982[b]) reported that *Narcissus* shoots wider than 8 mm could also be split into four quarters. This suggests that the number of parts into which a bulblet can be divided is limited by its size.

Shoots derived from *in vitro* grown bulblets arise from multicellular meristems - much like they do from twin-scales - in several species, including *Allium* (HUSSEY & FALAVIGNA, 1980; cited by HUSSEY, 1982[b]). According to Hussey (1982[b]), this may be a general phenomenon in bulbs, in which case propagation through bulblet division would give the same degree of genetic uniformity as from twin-scales.

While it is not always possible to "decapitate" the main shoot as in dicotyledons, the growth of inhibited lateral buds may be released by adding cytokinins to the medium. In bulbs and corms, the axillary meristems are located close to the basal plate which is a dome-shaped and reduced shoot. The basal plate is in direct contact with the medium and thus the

cytokinins are readily accessible (HUSSEY, 1976[a]).The involvement of cytokinins in apical dominance is a well known phenomenon and has been demonstrated in a number of dicotyledons (SACHS & THIMANN, 1964; cited by HUSSEY, 1976[a]) and in many bulbous species (HUSSEY, 1976[a]). Normally a single shoot is produced. However, when this is supplied with BA, the shoot branches (HUSSEY, 1976[a]). While species belonging to the Iridaceae are quite sensitive to cytokinins, those of the Liliaceae and Amaryllidaceae require much higher concentrations of BA to promote branching (HUSSEY, 1976[a]). For example, *Gladiolus*, *Iris*, *Sparaxis* and *Schizostylis* required 0.03 - 0.12 mg ℓ^{-1} BA but *Freesia* plantlets needed 0.5 mgℓ^{-1} BA and above to promote branching (HUSSEY, 1976[a]). Levels of 2.0 mg ℓ^{-1} and higher were found to cause branching in *Lilium*, *Narcissus* and *Hyacinthus* (HUSSEY, 1976[a]) without plantlet distortion or callus production occurring.

In the present study, *de novo* shoot initiation was enhanced by placing the divided bulblets on media containing cytokinins. Similarly, the differentiation of adventitious bulb scales from *Lilium* bulblets grown *in vitro* increased markedly when kinetin was added to the medium (TAKAYAMA & MISAWA, 1983). Shoot initiation could be increased by the addition of kinetin or thidiazuron, alone, when culturing *Scilla natalensis* (McCARTAN & VAN STADEN, 1998).The addition of BA to the medium is also reported to improve plantlet yield in *Agapanthus* (HUSSEY, 1980), *Alstroemeria* (HUSSEY, 1980), *Gladiolus* (HUSSEY, 1978; DANTU & BHOJWANI, 1995), *Freesia* (HUSSEY, 1980) and *Nerine* cultivars (CUSTERS & BERGERVOET, 1992). Here too, the numbers of new plantlets increased as the cytokinin level increased. In the work carried out by Hussey (1976[a]) an increase in the level of BA was shown to improve the rate of branching. Significantly higher levels, however, resulted in leaf distortion and root inhibition.

The inclusion of auxin in the medium is generally avoided because it stimulates callus production (HUSSEY, 1976[a]) or may reduce the frequency of shoot initiation, sometimes substantially (McCARTAN & VAN STADEN, 1998). Hussey (1976[a]), however, recognized that it could modify the concentration of cytokinin used if applied at the appropriate concentration, especially in the Liliaceae and Amaryllidaceae. That is why in species with strong apical dominance, either high cytokinin levels or a combination of auxin and cytokinin are used e.g. *Narcissus* (HUSSEY, 1976[a], 1980 and 1982[b]), *Hyacinthus* (HUSSEY, 1980) and *Nerine* (HUSSEY, 1980). The ratio of cytokinin to auxin is kept high for bulbs like *Narcissus* (HUSSEY, 1980 and 1982; SQUIRES & LANGTON, 1990; CHOW *et al.*, 1992[b]; BERGOÑÓN *et al.*, 1996) and *Nerine* (HUSSEY, 1980) as well as *Crinum macowanii* (SLABBERT *et al.*, 1993) and *Eucomis* spp. (AULT, 1995). In *Narcissus* cultivar tissue, for example, shoots are induced when the molar ratio of cytokinin to auxin is approximately 10:1 (SEABROOK *et al.*, 1976). In *Eucomis* species the number of shoots per microshoot explant increased as the amount of BA increased relative to that of NAA (AULT, 1995). Other workers have shown that

auxins, like NAA, and cytokinins lead to a proliferation of *in vitro* shoots. This has been demonstrated in *Hippeastrum* (HUSSEY, 1980) and *Nerine bowdenii* (PIERIK & IPPEL, 1977). According to Kukulczanka and Kromer (1988) both auxins and cytokinins are essential for bulblet production for if no growth hormones were used, bulblet production decreased during successive subcultures of *Vallota purpurea*.

The effect of different BA and NAA concentrations on the formation of secondary plantlets from *C. macowanii* bulblets showed that the greatest numbers were initiated in the absence of plant growth regulators (SLABBERT *et al.*, 1993). This has also been reported for *Hippeastrum* (SEABROOK & CUMMING, 1977; PIERIK *et al.*, 1990), *Scilla natalensis* (McCARTAN & VAN STADEN, 1998) and various other bulbs (YANAGAWA & SAKANISHI, 1980; cited by SLABBERT *et al.*, 1993).

Secondary shoots of *C. moorei* developed both in the light and dark. Those of *Gladiolus* showed better elongation in the light (DANTU & BHOJWANI, 1995). Bulblet formation from *in vitro* grown bulbs of *C. moorei* occurred only in the light. Light, therefore, is a requirement for bulblet development from twin-scales, as described earlier in this work, as well as from culture-derived bulblets. Regenerated bulblets of *Hippeastrum* hybrids are also routinely subcultured in the light (PIERIK *et al.*, 1990). Leaf development, rooting and bulbing of *Nerine bowdenii*, however, were much better when explant culture took place in the dark (PIERIK & IPPEL, 1977).

CONCLUSIONS

Provided cytokinins are used at the appropriate level i.e. without callus formation, they can be effectively used to enhance shoot multiplication from *in vitro* bulblets. The continuous multiplication of bulblets from *in vitro* material is important for horticultural practice since it can be used to increase virus-free stocks and store plant material indefinitely in culture.

Chapter 5

TRANSFER TO THE NATURAL ENVIRONMENT

INTRODUCTION

The final stage in the micropropagation scheme involves planting out the propagules. The success with which this can be achieved is an important element in determining the effectiveness and cost of micropropagation (SQUIRES *et al.*, 1991). In the commercialization of tissue cultured *Gladiolus* plants, for example, it is a "major bottleneck" (ZIV, 1979). Yet this is an area that has received limited attention (VAN AARTRIJK & VAN DER LINDE, 1986) with little known of the factors that influence the transplantation process.

Plantlets must first be rooted as this facilitates their transfer to the soil (STEINITZ & YAHEL, 1982; cited by CHOW *et al.*, 1992[a]) and allows active growth to continue (ILAN *et al.*, 1995). Monocotyledons, in particular, are known to root spontaneously in culture (YEOMAN, 1986) although auxins may be added to the final medium to accelerate root initiation (DREWES & VAN STADEN, 1994). Active charcoal is used to improve root growth where light intensities are high (ZIV, 1979).

Growth *ex vitro* not only depends on adequate root development but also on the type of propagule used. This is because, in many cases, the establishment of *in vitro* plants in non-aseptic conditions may cause problems (ZIV, 1979). Generally bulblets, rather than shoots, are used for planting *ex vitrum* as they improve plantlet survival (CHOW *et al.*, 1992[a] and 1992[b]); are easily transportable and can be mechanically handled (YEOMAN, 1986). They can also be sown like seeds at any time of the year whereas direct planting i.e. of rooted shoots, is seasonal since there are limited possibilities for storage. Their successful establishment, however, depends on the size and weight of the bulblets (SQUIRES & LANGTON, 1990) and, in some cases, cold treatment to break dormancy (SQUIRES & LANGTON, 1990). Other treatments involve the use of high temperatures and red light (VAN AARTRIJK & VAN DER LINDE, 1986). The inclusion of activated charcoal in the medium may also improve bulblet weight (SQUIRES *et al.*, 1991) and thus influences bulblet survival upon transfer to the soil (PECK & CUMMING, 1986). Other factors known to be important are media strength, sucrose concentration and light (SQUIRES *et al.*, 1991). Another approach sometimes used is to

induce dormancy so that tissue culture products can be stored for long periods (ILAN *et al.*, 1995). It may even be important for the acclimation of some species (ILAN *et al.*, 1995).

Finally, special care must be taken of the propagules when they are transferred to greenhouse conditions, that is, until they become autotrophic (ZIV, 1997). Conditions conducive to acclimatization include a relatively high humidity and low light intensities (KIM & DE HERTOGH, 1997; ZIV, 1997).

MATERIALS AND METHODS

Since the bulblets readily formed roots, they were transferred directly to *ex vitro* conditions without passing through a rooting stage. All traces of agar were washed from the plantlets after removing them from the culture vessel. They were then dipped in a fungicide solution containing Benlate (0.2%) and Dithane 45™ (which was used as directed i.e. 15 g per 10ℓ / 0.15%) in order to prevent subsequent infection. Bulblets were then planted in a potting mix composed of fine composted bark. This was sterilized so that the bulbs would not rot. The bulbs were then placed in the mist house to acclimatize. After six months they were removed from their pots and transferred to larger pots containing peat, bark and soil in a 1:1:1 ratio. To determine the effect of bulblet size on growth *extra vitrum*, bulblets were divided into three class sizes, namely: 3 - 4 mm, 5 - 6 mm and 7 - 9 mm (Figure 5.1). Bulblet diameter, the length of the longest leaf and the number of roots were recorded. The mass of each bulblet was recorded before planting out and again after 25 weeks. This was used to calculate the Growth Index (G.I.) as follows: $FW_2 - FW_1 / FW_1$ (ILAN *et al.*, 1995).

RESULTS

None of the bulblets appeared to be dormant at the time of planting out (Figure 5.2). The bulblets acclimatized readily in the mist house and showed continued leaf growth (Figures 5.2 and 5.4). Transplantation success was 88%. Bulblets that were 7 - 9 mm in diameter (the largest bulbs) at planting out grew best of all (Table 5.1 and Figure 5.3). On average, their growth index was 3.3 compared to 3.0 for the smallest bulblets and 2.2 for the bulblets with a diameter of 5 - 6 mm. After 25 weeks, the mean diameter of these bulblets was significantly higher than those in the other two size classes. The mean number of roots also differed significantly between the largest and smallest bulblets.

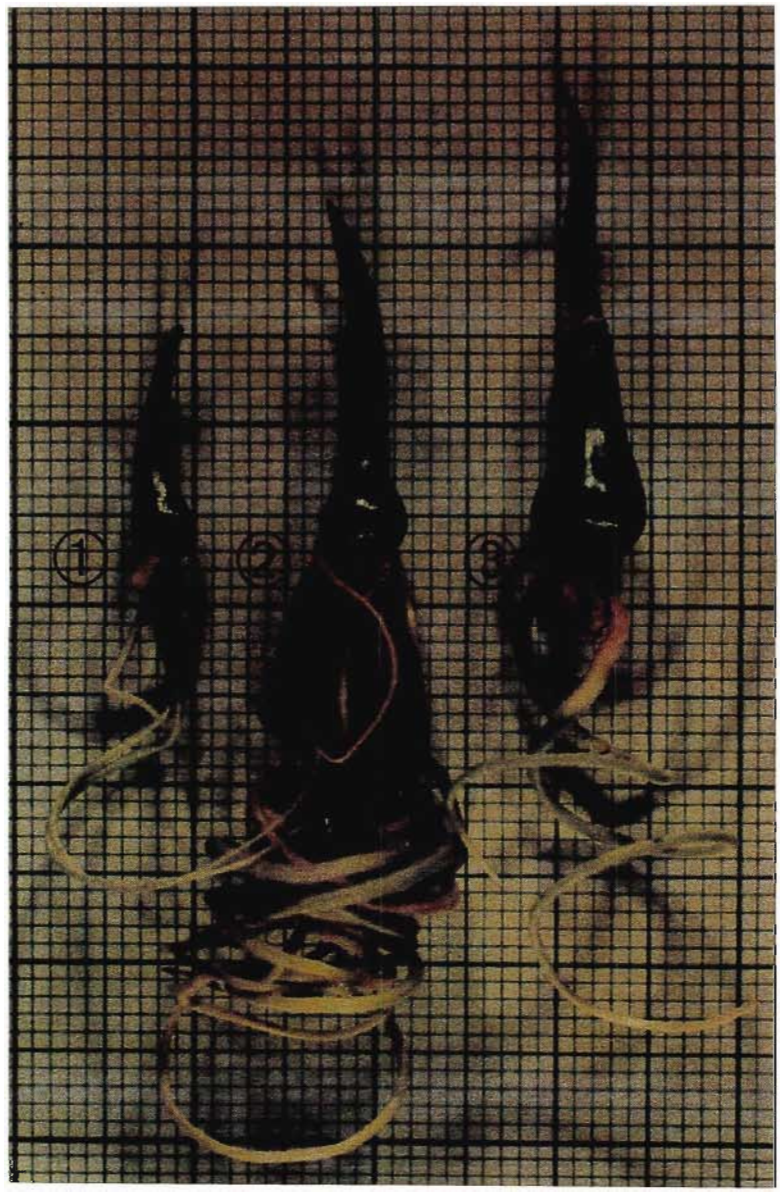


Figure 5.1: Different size classes of bulblets
1. 3 - 4 mm
2. 5 - 6 mm
3. 7 - 9 mm

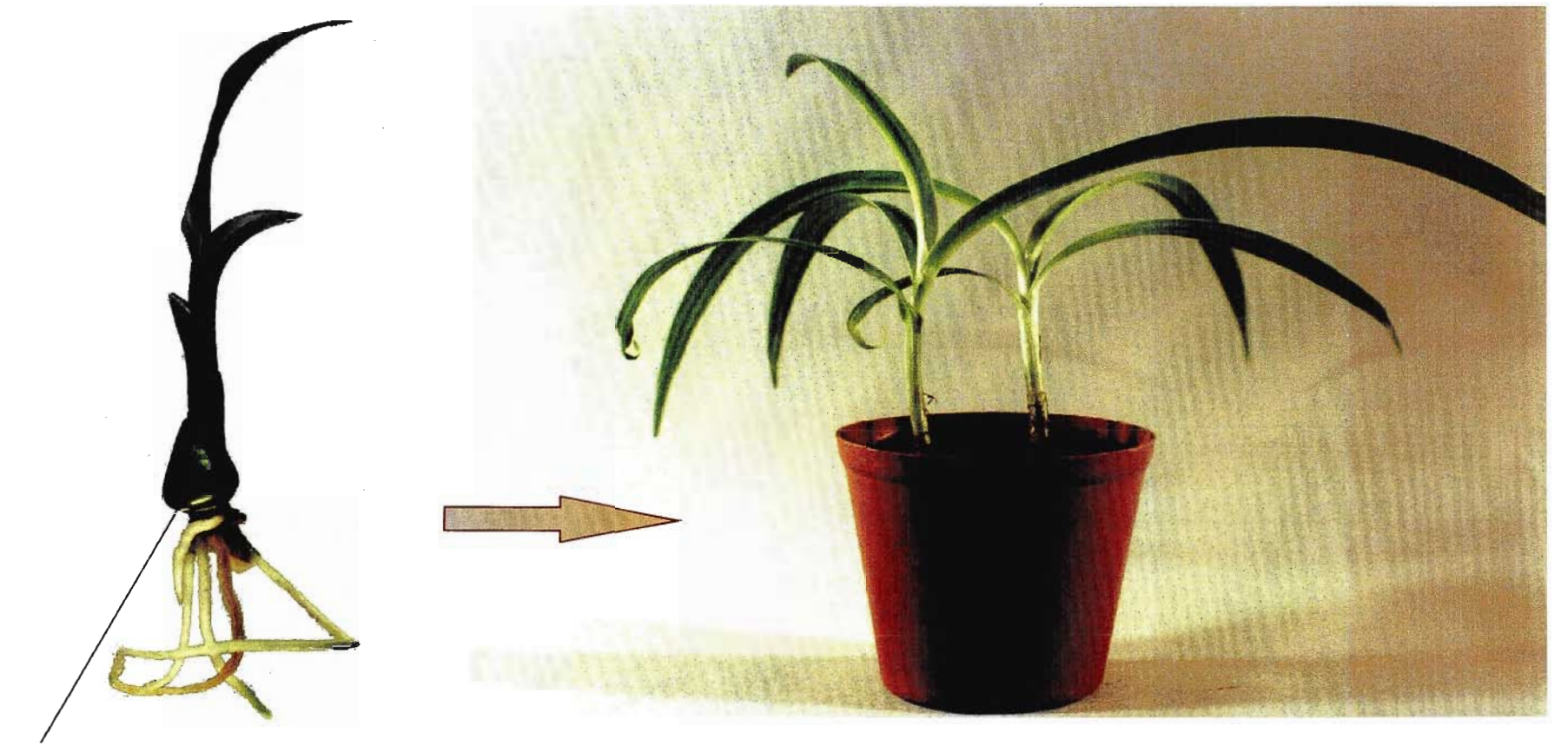


Figure 5.2: Transfer of bulblets to the soil

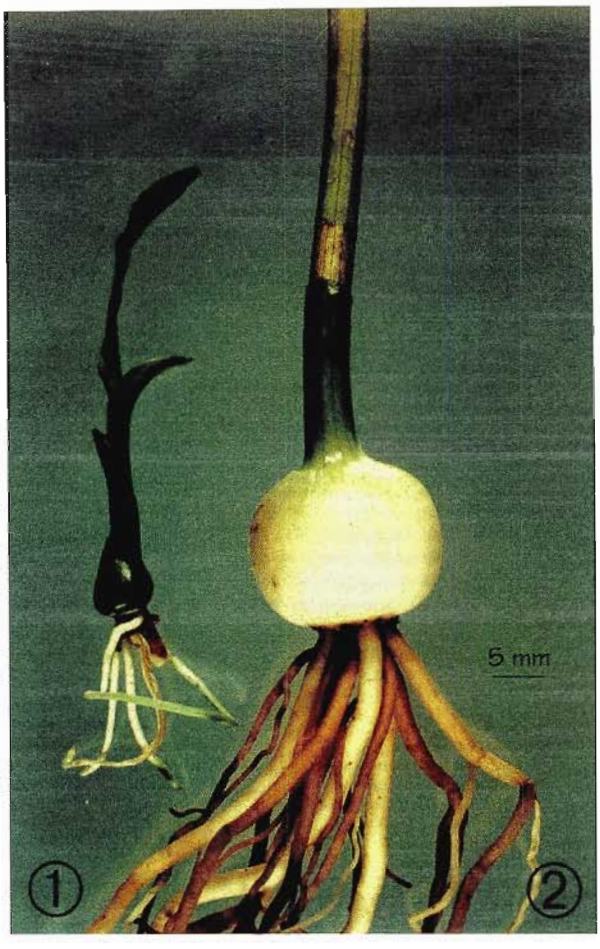


Figure 5.3: A comparison of bulblet size ① at planting out and ② after 25 weeks

Figure 5.4: *In vitro* grown bulblets successfully established in the greenhouse

Table 5.1: The effect of bulblet size on growth *ex vitro* (after 25 weeks).

Bulblet size class (mm)	Trans-plantation success (%)	G.I. ($FW_2 - FW_1 / FW_1$)	Mean bulblet diameter (mm)	Mean length of longest leaf (mm)	Mean number of roots
3 - 4	75	3.0^a	9.7^a	202^a	4.0^a
5 - 6	90	2.2^a	10.8^a	178^a	5.6^{ab}
7 - 9	90	3.3^a	14.0^b	218^a	7.4^b

Different letters show significant differences between treatments at the 5% level (ANOVA).

DISCUSSION

In vitro grown and non-dormant bulblets of *C. moorei* transplanted well with an overall success rate of 88%. The few losses that were encountered may have been due to the death and subsequent decay of the bulblets, especially those that had grown inverted in the tissue culture medium. De Bruyn and Ferreira (1992) reported success with *Gladiolus* corms as well, while survival for a range of *Narcissus* cultivars was recorded at 61.2% (SQUIRES & LANGTON, 1990), 47.8 - 81.2% (SQUIRES *et al.*, 1991) and at 80 - 90% by Steinitz and Yahel (1982). For *Gethyllis linearis* bulblets, the process of hardening-off also proved successful with 90% survival (DREWES & VAN STADEN, 1994). There appears to be some consistency in the success rate of bulblet transplantation although, as Squires *et al.* (1991) suggest, this may be cultivar dependent.

Poor post-transplanting growth has been attributed to the early onset of dormancy - usually marked by a brief foliar phase (HANKS, 1993; In: DE HERTOGH & LE NARD, 1993) - and sensitivity to high humidities and fungal attack, particularly among fleshy bulbs like *Boweia volubilus* (HANNWEG *et al.*, 1996). Plants removed from tissue culture lose excessive amounts of water because vascular development may be incomplete and epicuticular waxes reduced (HUGHES, 1981). Acclimatization and normal growth, however, can be achieved, first by satisfying any cold requirement of dormant bulbs (HUSSEY, 1980) and then using twice-daily mist sprays rather than tightly covering the potted plants in plastic bags (HANNWEG *et al.*, 1996). Improved survival was recorded for *Boweia volubilus* plants under these conditions (HANNWEG *et al.*, 1996), which possibly also contributed to the successful growth of *C. moorei* under ambient conditions. Growth after tissue culture is also influenced by bulblet size. For example, *Boweia volubilus* bulblets smaller than 4 mm in diameter died within 5 - 7 days after planting out. Only the large bulbs survived and produced mature plants (HANNWEG *et al.*, 1996). In the case of tulip, small bulblets from tissue culture did not sprout, while in lily, fast growth occurred in larger bulblets (LANGENS-GERRITS *et al.*, 1997). Thus, high rates of survival may be attributed to the large size of bulblets. In other geophytes there may be a further need for bulblets which have at least one leaf and two or more roots e.g.

Narcissus (STEINITZ & YAHEL, 1982; CHOW *et al.*, 1992[a]; LANGENS-GERRITS & NASHIMOTO, 1997) where it was noted that small bulblets with only one leaf and no roots survived comparatively poorly *ex vitro* (STEINITZ & YAHEL, 1982). In addition, lily bulblets must have formed a stem with several leaves rather than leaf-bearing scales (LANGENS-GERRITS *et al.*, 1997). Squires and Langton (1990) established that bulbil weight was critical in that it affected plantlet survival upon transfer to the soil. Optimal survival was achieved when larger bulblets, with a mass of more than 0.2 g, were used. According to Steinitz and Yahel (1982), *Narcissus* bulbs must have developed a bulblet of 250 - 300 mg fresh weight prior to planting in open field plots. This corresponds to a bulblet size of about 10 mm in diameter. Custers and Bergervoet (1992) used plantlets with a basal diameter of 7 mm for transplanting which they found to be very successful. The response of *C. moorei* bulblets also indicated that larger bulblets performed better in the soil. Although these were of a similar size, their mass at planting out was considerably higher, averaging 1.146 g. Bigger bulblets form mature meristems, better than smaller bulblets (BOONEKAMP, 1997). Gerrits *et al.* (1996; cited by BOONEKAMP, 1997) maintain that the maturation of the meristem is of greater significance than bulblet size for successful growth *ex vitro*. It may also have something to do with the abundance of the root system among larger bulbs. Steinitz and Yahel (1982) claimed that this was a prerequisite for the successful growth of bulbils. Poorly developed roots were also thought to contribute to the poor survival of transplanted *Gladiolus* plants (ZIV, 1979).

The Growth Index for the largest bulblets was higher than that for bulblets in the other two size categories. Since their mean fresh weight was higher at the time of planting out, nutrient depletion may have slowed the growth of the smaller bulblets. Squires *et al.* (1991) suggest that "weight increase is, at least to some extent, determined by the predisposing effect of initial bulbing shoot weight but (that) the mechanism underlying this is unknown".

CONCLUSIONS

"Proper morphogenetic treatments during tissue culture can accelerate further growth" (BOONEKAMP, 1997). In the case of *C. moorei*, bulblet size affected growth *extra vitrum*. This implies that it is first necessary to establish bulblets with a mean diameter of 7 - 9 mm in culture, before these can be successfully transplanted and grown on in the natural environment. In general, plantlets produced *in vitro* appear to be morphologically identical to the mother plants.

Chapter 6

PLANT GROWTH AND MORPHOGENESIS IN LIQUID CULTURE

INTRODUCTION

Limitations of micropropagation

"*In vitro* mass propagation methods today are still characterized by a high input of manual labour and low degree of automation" (PREIL, 1991). The demand for ornamental bulbs makes the labour intensive operations and equipment of agar cultures a costly proposition (TAKAHASHI *et al.*, 1992). Ziv (1989) estimates that 70 - 80% of the final cost of *in vitro* produced material is due to manual handling alone. Not only does the high cost limit expansion in terms of worldwide use (LEVIN *et al.*, 1988) but also the extension of the technology to other species (ZIV, 1995) which is currently used only for high value ornamental crops (ZIV, 1997). The technique is uneconomical for geophytes (ZIV, 1989).

Compared to micropropagation in liquid culture, growth of the propagules is considerably slower on solid media (ZIV, 1989; WATAD *et al.*, 1996). This is because the physical nature of the growth medium influences the rate at which cultures grow and multiply (WATAD *et al.*, 1996). Factors associated with gelling agents, and not necessarily liquid culture, include: gel matrix (DEBERGH *et al.*, 1981 and DEBERGH, 1983; cited by DESAMERO *et al.*, 1993); decreased water potential and diffusion rate (GEORGE, 1993) and; elemental alterations to the basal medium through the release of nutrients from agar (DESAMERO *et al.*, 1993). Agar, which is the most common support, may contain inhibitory substances (certain brands) that prevent morphogenesis (WATAD *et al.*, 1995); result in the selective or restricted absorption of compounds, both nutrient and growth regulatory, from the medium (GEORGE, 1993) and; slow diffusion of toxic exudates away from the explant (SIMMONDS & WERRY, 1987; WATAD *et al.*, 1996). Another likely cause of reduced growth on agar gels may be the immobilisation of the enzyme invertase released from the culture tissues as this would result in reducing the availability of glucose and fructose (GEORGE, 1993). Added to this is the poor survival rate *ex vitro* (ZIV, 1992[b]). Furthermore, the use of gelling agents precludes automation (ETIENNE *et al.*, 1999).

Why use a liquid system?

Plant culture in liquid media takes place either in agitated vessels or bioreactors (SMART & FOWLER, 1984; cited by ZIV, 1995). The method has traditionally been used only for growth of highly dispersed and non-morphogenic cell lines or embryogenic cultures (LEVIN *et al.*, 1988). It is however, proving popular, especially in the propagation of both bulbous and cormous plants (BERGOÑÓN *et al.*, 1992).

Labour-intensive manipulations associated with replenishing media for agar cultures, transferring propagules and vessel cleaning, are simplified, while handling costs and requirements for space, often consumed by large numbers of individual vessels, are reduced (LEVIN *et al.*, 1988; ZIV, 1990[a]; ETIENNE *et al.*, 1999). For geophytes the technique is easier to manipulate than subculturing individual bulb scales which are too small to separate (TAKAYAMA & MISAWA, 1983). This effectively shortens the time for subculturing as explants do not need to be positioned on the medium; medium changes can be effected by simple transfer and sterilisation performed by ultrafiltration (ALVARD *et al.*, 1993). Operator-associated contamination is thereby reduced (WATAD *et al.*, 1999).

The requirements for growth in a liquid system can be more precisely defined than is presently possible with conventional *in vitro* techniques (PREIL, 1991). In contrast to agar media of static cultures, there are no gradients in the media of liquid cultures (BERGOÑÓN *et al.*, 1992) with the result that culture conditions are more uniform (ETIENNE *et al.*, 1999). This includes pH which exhibits greater stability in liquid media. There is, consequently, an increase in the availability of water and dissolved substances (DEBERGH, 1983; cited by ALVARD *et al.*, 1993). There is also better contact between the plant biomass and medium (ZIV, 1995) because of the "bathing" effect (SIMMONDS & WERRY, 1987). The improved growth response with liquid feeding is perhaps a result of both increased availability of nutrients and dilution or washing away of toxic substances (SIMMONDS & WERRY, 1987; AITKEN-CHRISTIE, 1991).

Growth and multiplication of plants *in vitro* occurs more rapidly in liquid cultures; either static or shaken (DESAMERO *et al.*, 1993; WATAD *et al.*, 1995 and 1996). For many ornamentals, the short-term effects of liquid culture include increased shoot height, leaf growth, multiplication and rooting (AITKEN-CHRISTIE, 1991). While in geophytes, liquid feeding serves, not only, to stimulate cell division in several "dividing zones", as shown in the bulb scales of *Lilium longiflorum* (TAKAHASHI *et al.*, 1992[a]), but also to maintain the differentiation ability within the meristems. This was not observed for bulb scales cultured on an agar medium (TAKAHASHI *et al.*, 1992[a]). A further advantage is the increase in the proliferation of buds (SIMMONDS & WERRY, 1987; BERGOÑÓN *et al.*, 1992; CHOW *et al.*, 1993; ZIV,

1990[a] and 1997). The size of *Narcissus papyraceus* buds in a liquid shake medium, for example, was greater than those clusters cultured in the same medium but on agar (BERGOÑÓN *et al.*, 1992). A rapid increase in biomass during successive subcultures (ZIV & LILIEN-KIPNIS, 1997[a]) has also been observed for bud clusters of *Ornithogalum dubium* in liquid culture. There was a four-fold increase in the first and eight-fold increase in the second subculture (ZIV & LILIEN-KIPNIS, 1997[a]).

In addition to the stimulatory effect of liquid culture on shoot proliferation, there is a marked stimulation of bulblet growth e.g. *Hyacinthus orientalis* (TAKAYAMA *et al.*, 1991[a]) and *Lilium* species and hybrids (ISHIBA *et al.*, 1992; TAKAYAMA & MISAWA, 1983; HARUKI & YAMADA, 1992; KAWARABAYASHI, 1993; TAKAYAMA & TAKIZAWA, 1994; cited by NIIMI *et al.*, 1997), including *Lilium rubellum* (NIIMI *et al.*, 1997). Doubling of lily bulblet growth occurred after 25 days on agar but only after 5 days in liquid culture (TAKAYAMA & MISAWA, 1983[b]). Bulb size and weight increased (NIIMI *et al.*, 1997) - by as much as ten-fold (TAKAYAMA *et al.*, 1991[a]). The faster rate of storage organ formation, as in gladiolus cormlets, resulted in "shortening the time for the development of corms having the required size for flowering" (ZIV, 1989). A similar response was observed in *Nerine sarniensis* cultured in liquid medium. Here too, bulblet development was enhanced such that the time normally required for bulblets to reach an appropriate size for transfer to the soil was significantly shortened. This also contributed to early flowering (VISHNEVETSKY *et al.*, 1997; cited by VISHNEVETSKY *et al.*, 2000); an advantage over micropropagation by conventional means where bulbs only flower after a few years.

Plantlets grown in liquid media are suitable for automation as spent media can be changed with fresh, automatically (AITKEN-CHRISTIE, 1991). They are also the most likely means of scaling-up in bioreactors (AMMIRATO & STYLER, 1985; ZIV, 1989 and 1992; cited by ILAN *et al.*, 1995); the advantages of which include: larger vessels, automation and lower costs owing to the reduction in manual handling (ZIV, 1989; ZIV, 1990[b]; TAKAYAMA *et al.*, 1991[b]; ZIV, 1997). "Automation and scaled-up liquid cultures for *in vitro* plant propagation are mandatory to overcome some of the limitations imposed by labour intensive and high production costs of existing conventional techniques" (ZIV, 1995). Since propagules must first be introduced into liquid culture (ZIV, 1995), it is important at this stage to design a medium that facilitates good nutrient uptake and aeration of the tissues; factors critical for automation (AITKEN-CHRISTIE, 1991). Scaling-up in larger volumes, as in tank cultures, has enabled bulblets to be produced on an industrial scale (TAKAHASHI *et al.*, 1992[a] and 1992[b]). To date, liquid cultures in bioreactors have been successful and efficient for *Lilium*, *Gladiolus*, *Nerine* and *Brodiaea* (TAKAHASHI *et al.*, 1992; ZIV, 1997). Various factors affect biomass production and the quality of the propagules, including: the type of bioreactor, method of aeration and circulation, agitation, shearing forces, the gaseous environment, pH, light,

temperature, media components, including plant growth regulators, rheology and osmoticum (TAKAHASHI *et al.*, 1992; ZIV, 1995). Airlift and bubble column bioreactors are preferred to stirred tank bioreactors because of their low shear properties (ZIV, 1995). They are useful for scaled-up micropropagation, somatic embryogenesis and hairy root culture (ZIV, 1995). Furthermore, liquid systems have the potential to be used in the production of pharmaceutically interesting compounds since these are often released into the medium (BERGOÑÓN *et al.*, 1992 and 1996; SELLÉS *et al.*, 1997) from which they may easily be recovered.

Pathways

Both embryogenic and organogenic pathways are used for large-scale liquid cultures (ZIV, 1995; ZIV *et al.*, 1995) although somatic embryogenesis has been the most commonly used route for morphogenesis in liquid culture, including bioreactors (LILIEN-KIPNIS *et al.*, 1992; ZIV *et al.*, 1994). Embryogenesis involves the development of bipolar meristems that ultimately form whole new plants, or perennating organs e.g. *Nerine* x mansellii (LILIEN-KIPNIS *et al.*, 1992). Provided that somatic embryos form freely in suspension cultures, the system offers advantages for mass propagation, that, up until now, have not been fully realised (PREIL *et al.*, 1988). Organogenesis is the induction of unipolar meristems that lead either to shoot or root formation (ZIV, 1997). Information is, however, limited on propagation through the organogenic pathway (LEVIN *et al.*, 1988; cited by ZIV, 1991; ZIV, 1995). To date, it has been employed in the micropropagation of ferns, *Pinus* and Poplar (woody species), *Philodendron*, *Begonia* (herbaceous plants) (ZIV *et al.*, 1994) and petaloid monocotyledons (KRIKORIAN & KANN, 1986; TAKAYAMA *et al.*, 1991[a] and 1991[b]; ZIV, 1989 and 1991; cited by ZIV *et al.*, 1995). The latter includes both bulbous and cormous species (LILIEN-KIPNIS *et al.*, 1992) like *Gladiolus* cv. Eurovision (ZIV, 1989), lilies (*Lilium longiflorum*) (ZIV, 1995) and *Ornithogalum dubium* (ZIV, 1997). In scaling-up, organogenesis presents fewer problems than cell cultures which form aggregations or foam (ZIV, 1995; ETIENNE *et al.*, 1999). As meristematic or bud clusters are less sensitive to shearing stresses, they may be used to circumvent the problems of foaming and high culture viscosity (ZIV, 1995). An added benefit would be the induction of storage organs, either in the liquid stage or after subculture to the hardening stage, as these can be easily transplanted, shipped or kept in storage (ZIV, 1997).

Physical and nutritional requirements

Growth of differentiated tissues in large-scale liquid cultures differs markedly from that of either microbial or plant cell cultures. There is, therefore, a "need to understand the requirements imposed on differentiated cells by large-scale culture of clusters, embryos or

buds" (ZIV *et al.*, 1994). Various factors have subsequently been shown to affect biomass production and the quality of the propagules in a liquid system (ZIV, 1995). These include: inoculum to medium ratio; cluster or organ size (ZIV *et al.*, 1994); media components; the gaseous phase in the culture vessel; temperature and light (ILAN *et al.*, 1995). In general though, most systems use variations of the same basal medium for growth on agar and in liquid culture (PREIL, 1991). "However, regulatory systems for the control of the internal environment and for defining the requirements for quality plant regeneration in bioreactors are still limited to a few crop species" (ZIV, 1995).

The problem of hyperhydricity

Undesirable phenomena, such as explant asphyxia (ALVARD *et al.*, 1993), morphological aberrations and hyperhydricity, are often encountered in liquid cultures (ILAN *et al.*, 1995; ZIV *et al.*, 1994) because the explants are submerged in the medium and / or vigorously aerated (ZIV, 1991). More often than not these are associated with the organogenic pathway. Multiplication is impeded by hyperhydricity (ZIV, 1995) which is manifested in abnormal shoots with vitreous leaves (ZIV, 1992) and abnormal stomata (ZIV, 1992). As a result, the shoots do not transfer readily *ex vitro*.

The problem can, however, be controlled by the medium osmoticum, which limits leaf expansion (ZIV, 1991), or overcome by the use of growth retardants, particularly those that inhibit gibberellin biosynthesis (ZIV, 1990[a]; ZIV, 1995). These result in reducing or inhibiting leaf expansion or in compact bud / meristemoid cluster formation (ZIV, 1992; ZIV *et al.*, 1994). In place of leaf development, shoots, because they are restricted to primordial tissue, form spherical organised structures (ZIV, 1999) or meristematic aggregates (ZIV, 1990[a]) that continue to divide actively (ZIV *et al.*, 1995). Not only does this obviate hyperhydration (vitrification) by preventing or reducing morphological and physiological aberrations, but automated biomass separation and inoculation onto agar is greatly facilitated (ZIV, 1992). Further development, acclimatization and preparation for transfer *ex vitro* requires a hardening stage. Meristemoid clusters of *Nerine* (ZIV, 1992), for example, were hardened by the addition of a second liquid layer (above the agar) and induced to form plants or storage organs.

Storage organ development may further reduce the problem of abnormal shoot morphogenesis during the proliferation stage, as shown in *Gladiolus* (DANTU & BHOJWANI, 1987; ZIV, 1979 and 1989; cited by ZIV, 1990[a]). By preventing leaf growth, growth retardants "force" the onset of storage organ formation (ZIV, 1989 and 1990[a]); provided there is sucrose in the medium (ZIV, 1990[a]). This obviates the need for acclimatization of plantlets derived from meristemoid clusters (ZIV, 1990[a]) and provides an efficient system for the storage and international transfer of storage organs (ZIV, 1990[a]). Several growth retardants have been

tested for their ability to limit growth. Of these, ancymidol (ANC) and paclobutrazol (PAC) were the most effective (ZIV, 1991). Both ANC and PAC are known to regulate shoot morphogenesis; enhance bud proliferation and corm production in *Gladiolus* liquid cultures (ZIV, 1989; STEINITZ & LILIEN-KIPNIS, 1989; cited by ZIV, 1992[b]; STEINITZ *et al.*, 1991); as well as regulate storage organ formation in other geophytes (ZIV, 1990[a]; ILAN, 1990; cited by ZIV, 1992[b]).

The problem of hyperhydricity may further be overcome using vented vessels with membrane rafts or floats or by partial immersion of the explants to ensure aeration (ALVARD *et al.*, 1993). To this end, inert absorbent substances e.g. filter paper, cellulose, rockwool etc., are used to maintain contact between the medium and the base of the explant (ALVARD *et al.*, 1993).

AIMS

Little is known about bulb regeneration in serial subculture. As this has important implications for the establishment of a mass propagation system, the multiplication of *C. moorei* bulblets during stage two was investigated further. Culture-derived bulblets of *C. moorei* are used for multiplication by cutting them into halves. This limits production. Furthermore, growth of the subcultured bulblets is slow. Liquid systems hold the most promise for large-scale culture and ultimately automation. For this reason, regeneration and growth of the bulblets were examined in solid and in liquid media with different degrees of support. The effects of cytokinins in inducing tissue proliferation and on morphogenesis during liquid culture was also studied, along with the ability of the morphogenic tissue to regenerate bulblets upon transfer to gelled medium supplied with either reduced levels of BA, high sucrose concentrations or activated charcoal. The aim was, therefore, to increase the rate of multiplication by inducing the formation of new and multiple shoots in liquid culture.

A COMPARISON OF DIFFERENT CULTURE PROCEDURES FOR THE MULTIPLICATION OF PROPAGULES

Introduction

Liquid cultures are frequently used for propagating ornamentals owing to the advantages of the system over conventional propagation practices in solid media. This is because of the higher growth rates achieved in liquid cultures and the more rapid response of the tissues to media manipulations and selection pressures (NAGASAWA & FINER, 1988). Sometimes it is preferable to place small explants on mechanical supports e.g. M- or U-shaped filter paper strips, which act like wicks (GEORGE, 1993). In this way nutrients are made available and the tissues are sufficiently aerated to prevent hyperhydricity.

Materials and methods

Adventitious shoot initiation from bulblets in different culture conditions

"Halved" 3 - 5 mm bulblets of *C. moorei* that had been grown under standard conditions were used. These were placed on: (1) solid medium, (2) filter-paper bridges in liquid medium and (3) liquid medium in 250 mℓ Erlenmeyer flasks and shaken on a rotary shaker (Faculty of Science Workshop, University of Natal Pietermaritzburg) at 104 rpm. The initiation medium used was a modified MS medium containing 10 mg ℓ^{-1} BA. A 10 mℓ volume of the medium was used for treatments 1 (solid) and 2 (liquid) while the flasks (treatment 3) contained 50 mℓ. The propagules were placed in the light at 25°C. Fresh weights were recorded by weighing tissues on a digital balance under sterile conditions in a laminar flow cabinet. The Growth Index (GI) was calculated as follows: $GI = (FW_2 - FW_1 / FW_1)$. The mean GI of 10 replicates is shown for the solid and liquid treatments. For the liquid-shake culture, three halved bulblets were placed in each of the five flasks. The GI, in this case, represents the mean for 15 samples. The results were analysed statistically, as outlined in Chapter 3. This procedure was followed for all subsequent experimental work.

Shoot growth and morphogenesis in different culture conditions

Shoots derived from "halved" bulblets subcultured to solid medium were placed on (1) solid medium, (2) filter-paper bridges in liquid medium and (3) liquid medium in 250 mℓ Erlenmeyer flasks and shaken on a rotary shaker (104 rpm.). The initiation medium used was a modified MS medium containing 10 mg ℓ^{-1} BA. The Growth Index was calculated as above.

Results

Adventitious shoot initiation from bulblets in different culture conditions

Growth and proliferation of propagules under constant submergence in liquid media was compared to that achieved for solid and other cultures supported by filter-paper bridges. The Growth Index for the shoots developing in the liquid-shake medium was higher than that for both the solid and liquid medium although the mean number of shoots produced in each explant was higher for those bulblets that had been placed on the solid medium (Table 6.1). These results were, however, not statistically significantly different. Nodular callus was present in the liquid-shake medium. In time, this increased in amount, proliferated extensively into meristemoids and became interspersed with shoots that developed indirectly from the callus (Figure 6.1).

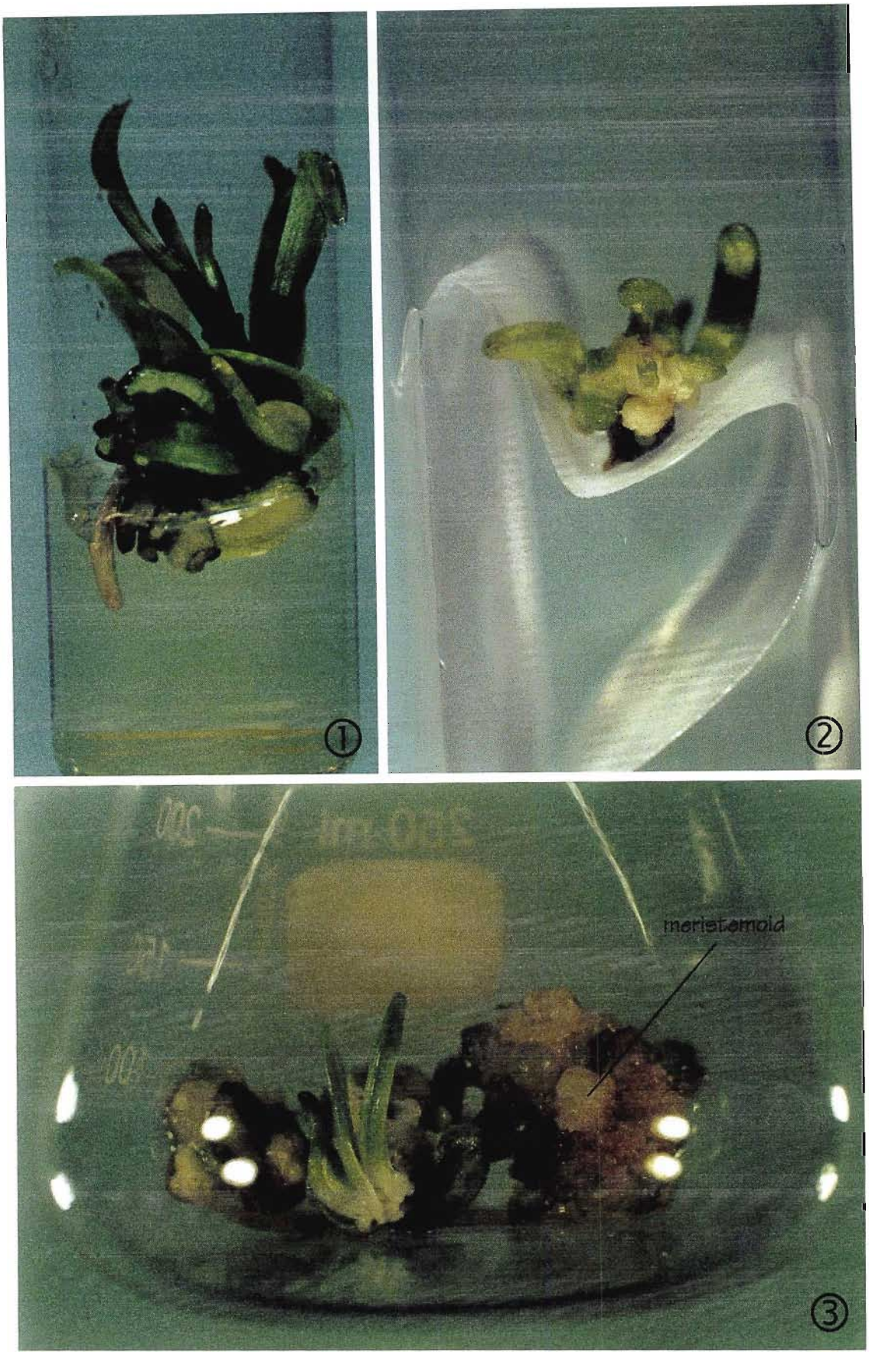


Figure 6.1: Adventitious shoot induction from bulblets in different culture conditions

1. Multiple shoots (solid multiplication medium)
2. Multiple shoots (liquid multiplication medium with filter paper bridge)
3. Multiple shoots and meristemoids (liquid-shake multiplication medium)

Table 6.1: The effect of media type on the development of secondary shoots from *C. moorei* bulblets

Type of medium	Growth Index (after 11 weeks)	Mean number of shoots / explant (after 11 weeks)
Solid	3.05[a]	2.30[a]
Liquid	2.06[a]	2.15[a]
Liquid-shake	3.62[a]	2.19[a]

Different letters show significant differences between treatments at the 5% level (ANOVA).

Shoot growth and morphogenesis in different culture conditions

The Growth Index for the liquid-shake medium was higher than that for both the solid and liquid medium and statistically significantly different to that achieved in static liquid culture. The mean number of shoots produced in each explant was, however, higher for those shoots that had been placed on the solid medium, although not significantly so (Table 6.2). Although there were fewer shoots in the liquid-shake medium, callus proliferation in this type of medium contributed to the higher Growth Index.

Table 6.2: The effect of media type on secondary shoot growth and shoot multiplication

Type of medium	Growth Index (after 3 weeks)	Mean number of shoots / explant (after 3 weeks)
Solid	1.68[ab]	4.0[a]
Liquid	0.82[b]	3.2[a]
Liquid-shake	2.94[a]	1.6[a]

Different letters show significant differences between treatments at the 5% level (ANOVA).

Discussion

The type of medium application i.e. solid or liquid, influenced the growth response of *Crinum moorei* shoot cultures as expected. Biomass increased preferentially in liquid-shake culture. There were also noticeable differences in morphogenesis. In response to agitation in a liquid medium, adventitious shoots developed into meristemoid clusters.

Liquid-shake cultures have successfully been developed for other bulbous plants like *Lilium*, *Begonia* (AITKEN-CHRISTIE, 1991), *Nerine* (ZIV *et al.*, 1994) and *Narcissus* (ZIV & LILIEN-KIPNIS, 2000). An agitated shake culture was also substituted for an agar-solidified system for the micropropagation of *Gladiolus* (ZIV, 1989). Mostly this is because of the improved regeneration response and rapid rate of propagule proliferation in liquid cultures. For example, bud explants of *Gladiolus* that were placed in liquid media and agitated, increased in size and

proliferated (ZIV, 1989) forming large bud aggregates without leaves (ZIV, 1990[a]). In the case of *Ornithogalum dubium*, the mass of bud clusters increased four-fold (2.5 - 10 g) after just four weeks in liquid culture (ZIV & LILIEN-KIPNIS, 1997[a]). Upon transfer to a bioreactor containing liquid medium, the initial inoculum of 15 g increased to 140 g (a growth value of 8.3) within 32 days (ZIV & LILIEN-KIPNIS, 2000). Discs of *Narcissus* scapes bearing 3 - 4 mm long buds produced more than 190 buds in a period of four weeks (ZIV & LILIEN-KIPNIS, 2000). Nodular tissues of *Nerine* increased in size and proliferated into rounded, compact and easily crumbled meristematic clusters when placed in liquid culture (ZIV *et al.*, 1994 and 1995). Their fresh weights increased four to six fold every 28 days over a prolonged period (ZIV *et al.*, 1994).

Additional benefits of liquid culture, as demonstrated by other studies, include: enhanced shoot or bulb scale production; fresh or dry weight gain and leaf formation. Increased efficiencies of liquid micropropagation systems are reported for *Begonia* species (NAKANO *et al.*, 1999) e.g. shoot proliferation and growth of *Begonia* x *tuberhydrida* Voss was stimulated by liquid-shake culture with significant differences in shoot numbers compared to treatments solidified with gellan gum. The regeneration of *Cucumis metuliferus* shoots also improved in a liquid system compared to semi-solid media (ADELBERG, 1998) with nine buds developing per cotyledon as opposed to only six on the solid medium. For *Aconitum napellus*, shoot multiplication occurred more rapidly in liquid shake culture (WATAD *et al.*, 1995) with more shoots developing in half the time than that recorded for solid media. Shoot tips of *Lilium* grew better in liquid MS medium than on agar (TAKAYAMA & MISAWA, 1983[a]). So did bulb scales; yielding numerous secondary bulb scales (TAKAYAMA & MISAWA, 1983[b]). Growth doubling time for bulb scales grown on agar medium was 25 days but only five in liquid-shake culture (TAKAYAMA & MISAWA, 1983[b]). Even though the number of watermelon (*Citrullus lanatus*) buds (unelongated shoot meristems) was comparable in liquid and solid media, tissue fresh weights were greater in the liquid system (DESAMERO *et al.*, 1993). A comparison of growth of banana shoots in liquid and gelled media showed that multiplication rates, dry weight gain and leaf development were superior in liquid media, especially when the shoots received a temporary immersion treatment (ALVARD *et al.*, 1993).

The benefits of a liquid-shake system can be attributed to the effects of agitation and bathing the tissues in the nutrient solution. Agitation enriches the medium with oxygen, ensures better mixing and absorption of the medium constituents and prevents clusters from settling (STUART *et al.*, 1987; cited by ILAN *et al.*, 1995); thus allowing for optimal growth (ZIV, 1995). Differences in the gaseous atmosphere may have contributed to improved growth in Erlenmeyer flasks, which was higher than that recorded for poinsettias grown in bioreactors (PREIL, 1991; cited by ZIV, 1995).

The availability of mineral nutrients depends on whether the medium is gelled or liquid (ZIV, 1995). Both the viscosity of the medium and aeration will affect the rate at which the constituents of the medium are absorbed (DEBERGH *et al.*, 1994; WILLIAMS, 1992; cited by ZIV, 1995). Since tissues in liquid media are better exposed to components of the medium, these are better absorbed (ZIV, 1995). The propagation of *Nerine samiensis* in liquid medium was enhanced because the cells absorbed nutrients and water immediately upon contact with the liquid (VISHNEVETSKY *et al.*, 2000). Takayama and Misawa (1983[b]) suggested that the improved growth of *Lilium* bulb scales in liquid-shake culture was because the tissues were in continuous contact with the medium. Explants in culture vessels placed on shakers have a greater surface area that is exposed to the medium. Agitation of the medium reduces the diffusion gradient between the medium and the explant with the result that growth and multiplication are increased (WATAD *et al.*, 1995). Apart from the increased availability of nutrients, it has also been suggested that toxic substances are effectively dispersed in the liquid phase (GEORGE, 1993) and therefore washed away when the liquid medium is gently agitated (AITKEN-CHRISTIE, 1991).

Studies involving shoot proliferation of watermelon in liquid and agar-solidified media indicated pH differences between the two types of media (DESAMERO *et al.*, 1993). The pH of the liquid medium was generally lower than that recorded in agar, which may have been influenced by hyperhydric tissues. The authors propose that the vitrified shoots, as a consequence, affected growth and nutrient uptake (DESAMERO *et al.*, 1993).

Proliferating tissues of watermelon in liquid culture were visibly more water soaked and turgid than tissues that had been grown on agar-solidified media (DESAMERO *et al.*, 1993). This was due to the fact that they contained proportionately more water. It is therefore possible that tissues immersed in liquid media take up more water. However, since the bud clusters of *C. moorei* did not appear water soaked or hyperhydric, their higher growth rates in liquid media were as a result of shoot proliferation rather than increased water absorption.

In contrast, poorer regeneration values were obtained for carnation shoots cultured in shaken liquid media because of vitrification, which was thought to inhibit morphogenesis, and damage to shoot primordia during agitation (WATAD *et al.*, 1996).

Poorer growth rates were recorded in liquid cultures employing filter paper bridges for the multiplication of *C. moorei*. The response of tissues grown on agar or filter paper supports varies from species to species (GEORGE, 1993). Carnation shoots grew less well on filter paper supports while *Leucospermum* buds did not survive on agar but had to be placed first on filter paper bridges for a period of three months (DAVIS *et al.*, 1977 and KUNISAKI, 1990;

cited by GEORGE, 1993). In *Hemerocallis*, *Delphinium* and *Iris*, growth was proportional to nutrient uptake for explants grown on filter paper bridges (LUMSDEN *et al.*, 1990). The absence of transpiration meant that nutrient uptake was via diffusion. This was thought to be promoted by diffusion gradients created by nutrient utilisation for growth (LUMSDEN *et al.*, 1990). Since the availability of nutrients is limited by how quickly they can diffuse through the filter paper, it is conceivable that this must be slower than nutrient uptake in liquid cultures where explants are in direct contact with the medium. Alvard *et al.* (1993) also suggest that the small surface area of banana explants in contact with medium-soaked supports (cellulose) may have limited the supply of water, carbohydrates and mineral nutrients such that the shoots were weakly developed. Other reports indicate that growth is slower in static liquid media because of a lack of oxygen; a major limiting factor to growth (ALVARD *et al.*, 1993). Thus, in order to achieve the benefits of liquid media without the disadvantages of hyperhydricity associated with submerged tissues, explants may be supported by polypropylene membrane rafts above the liquid. This has been advocated for orchid, tomato and begonia shoot cultures in order to enhance growth (DESAMERO *et al.*, 1993).

The proliferation of buds into meristemoid clusters (without leaves) appears to be a common phenomenon in shaken or rotated liquid cultures e.g. *Gladiolus* (ZIV, 1989) and *Nephrolepis exaltata* (ZIV, 1991). The bud aggregates have no leaves but are able to develop later into storage organs (ZIV, 1989). Garlic tissues in suspension culture proliferated as nodular clumps (NAGASAWA & FINER, 1988). These were less organised than the starting material which consisted of shoot buds. Callus-like tissue interspersed with nodular regions of *Nerine* x mansellii was also reported to proliferate in liquid media, yielding compact, but friable meristematic clusters (LILIEN-KIPNIS *et al.*, 1992). Each cluster was made up of several nodules arranged concentrically. Similar structures were reportedly also produced in poplar and Radiata pine (ZIV *et al.*, 1994) where they were referred to as "nodules" (AITKEN-CHRISTIE *et al.*, 1988 and McGOWAN *et al.*, 1988; cited by ZIV *et al.*, 1994). Aggregates of meristematic tissue ("nubbins") also occur in daylily (KRIKORIAN & KANN, 1986; cited by ZIV *et al.*, 1994). But in *Gladiolus*, *Philodendron* and ferns, they are called "meristematic clusters" (ZIV *et al.*, 1994) or protocorm-like bodies in the case of *Gladiolus* and *Brodiaea* (ZIV, 1999). The nodular clusters in *Gladiolus* later developed into meristematic centres interspersed with parenchyma cells and large air spaces (ZIV, 1990; cited by ZIV *et al.*, 1994; ZIV *et al.*, 1995). Generally the clusters are made of densely packed cells that are organised into actively dividing meristematic centres that have morphogenetic potential (ZIV, 1999). It is thought that plant growth regulators, in combination with continuous agitation, may influence this response, as meristematic clusters were not a feature of cultures grown on media solidified with agar (this study). Instead, the bud primordia proliferated adventitiously giving rise to multiple shoots. Generally, morphogenesis proceeds normally or preferentially in agar

cultures. It may even improve (GEORGE, 1993). Watermelon buds, for example, exhibited better elongation in agar-solidified media (DESAMERO *et al.*, 1993). The fact that an agar-solidified medium has a lower (more negative) water potential than the same medium without agar, may partly explain why the morphogenetic response of tissues on solid medium is better than in its liquid equivalent (GEORGE, 1993).

Nodules of meristematic tissue are ideal for automation since they proliferate in liquid media and, because they are relatively homogenous, can be mechanically divided or macerated (ZIV, 1989), and separated and screened for uniformity (AITKEN-CHRISTIE, 1991). They are used successfully in the propagation of garlic (*Allium sativum*) and daylily (*Hemerocallis*) (AITKEN-CHRISTIE, 1991). Suspension cultures in the early stages of morphogenesis may also be a good source of protoplasts, which for garlic, was desirable in that they could potentially be used for somatic hybridization and gene transfer (NAGASAWA & FINER, 1988).

FACTORS AFFECTING THE MULTIPLICATION OF PROPAGULES IN LIQUID CULTURE

Introduction

The increase in plant biomass during the proliferation stage in liquid cultures is influenced by the size of the inoculum and medium constituents (ZIV, 1991). Generally, plant growth regulators that are used for agar culture are adapted for liquid culture (ZIV, 1995). They are, however, more effective in controlling growth in liquid culture, as the tissues are in direct contact with the medium (ZIV, 1995). This deserves further research (ZIV, 1995).

Materials and methods

Shoots derived from "halved" bulblets were transferred to 100 mℓ Erlenmeyer flasks containing 50 mℓ liquid MS medium with either 0, 0.1, 1 or 10 mg ℓ^{-1} BA. The flasks were placed on a rotary shaker (120 rpm) in the light at 25°C.

Results

Shoot cultures of *C. moorei* proliferated rapidly in liquid culture and formed clusters of shoot apices. As it was impossible to count these shoots, the weight of the tissue was determined in order to monitor the growth value over time.

Biomass increase and shoot growth were superior in liquid MS media without BA (Table 6.3). This was, however, not significantly different, statistically, to the other treatments employed.

In addition, 20% of the shoots developed bulblets, whereas none were recorded in media containing BA. Shoots in liquid-shake media containing high concentrations of BA developed meristematic clusters. The concentration of BA used thus influenced the morphogenetic response of the tissues. At lower levels, or in its absence, bulblets were induced, whereas at higher concentrations (10 mg ℓ^{-1}), meristematic clusters (meristemoids) formed (Figure 6.2).

Table 6.3: The effect of BA concentration on shoot growth (after 8 weeks)

BA concentration (mg ℓ^{-1})	Growth Index	Morphogenic response
0	6.63[a]	shoots and / or bulblets
0.1	3.41[a]	meristematic clusters
1	4.75[a]	meristematic clusters
10	5.82[a]	meristematic clusters

Different letters show significant differences between treatments at the 5% level (ANOVA).

Discussion

In the control (lacking the cytokinin BA), growth indices were high and spontaneous bulblet development for *C. moorei* was recorded. When bulb scales were used for propagating *Lilium* in liquid-shake culture, these grew rapidly in media without kinetin (TAKAYAMA & MISAWA, 1983[a]), although the optimum concentration did vary between species. Bulb scales also multiplied in media containing kinetin together with 30 g ℓ^{-1} sucrose (TAKAYAMA & MISAWA, 1983[a]). A *Begonia* hybrid grown in liquid-shake culture produced elongated shoots without plant growth regulators more efficiently than when hormones were included. However, the total number of shoots improved using a liquid medium containing NAA and BA (NAKANO *et al.*, 1999). Upon transfer to a second medium with no plant growth regulators, the small shoots could be induced to elongate (NAKANO *et al.*, 1999).

Corms differentiated spontaneously in liquid cultured *Gladiolus* plantlets when the medium was devoid of plant growth regulators (DANTU & BHOJWANI, 1987; cited by STEINITZ & LILIEN-KIPNIS, 1989). This was achieved only sporadically in other studies (STEINITZ & LILIEN-KIPNIS, 1989) where it was reported that corms were very small after incubating them for a month in the liquid-shake medium. The production of *Gladiolus* cormlets in liquid medium was attributed to the inhibition of leaf growth by including growth retardants, such as PAC, in the medium (ZIV, 1989). Other geophytes e.g. potato tubers, also require endogenous cytokinins for tuberization (STEINITZ & LILIEN-KIPNIS, 1989). In the work on corm production in *Gladiolus* (STEINITZ & LILIEN-KIPNIS, 1989), however, corm formation

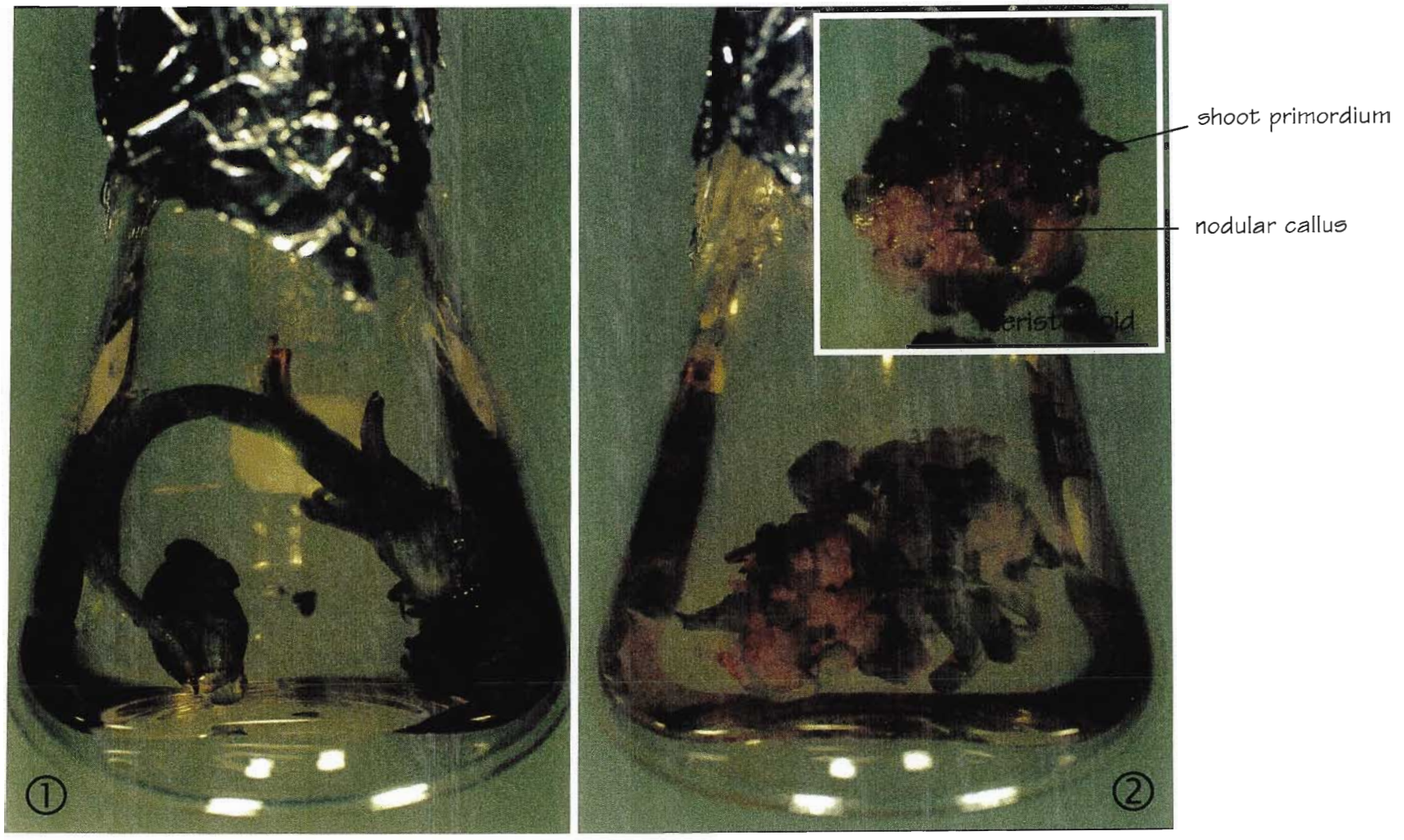


Figure 6.2: The effect of BA concentration on shoot morphogenesis in liquid-shake culture

1. Bulblets - medium without BA
2. Meristemoids - medium supplemented with 10 mg ℓ^{-1} BA

was completely inhibited by BA. The authors concluded that in this case, BA could not be used as an agent to induce corm formation (STEINITZ & LILIEN-KIPNIS, 1989).

Low concentrations of BA (0.1 and 1 mg ℓ^{-1}) had an inhibitory effect on the growth value of *C. moorei*. In some respects this is similar to the response of meristematic clusters of *Nerine* x mansellii in liquid media containing PAC (LILIEN-KIPNIS *et al.*, 1992).

High concentrations of BA (10 mg ℓ^{-1}) resulted in improved growth compared to the lower levels used. In *Nerine*, the presence of PAC had a similar effect in that it increased the biomass of meristematic clusters (ZIV *et al.*, 1994) even more so when it was used for shorter periods (10 days); just like *Gladiolus* (ZIV *et al.*, 1994). This may be due to its ability to enhance adventitious as well as axillary bud development (ZIV *et al.*, 1994), by inhibiting the growth of the apical bud, and its action as a promotive bioregulator for meristematic cluster formation (ZIV *et al.*, 1995). BA and NAA were used for shoot development in liquid-cultured *Gladiolus* (ZIV, 1989; ZIV, 1990[b]). Their addition to the growth medium was thought to have enhanced adventitious bud proliferation (ZIV, 1989). Increasing the level of BA also led to a gradual increase in shoot proliferation for cultures of *Aconitum napellus* (WATAD *et al.*, 1995). In contrast to the control, however, there was a reduction in biomass of *C. moorei* when high levels of the cytokinin BA were included in the medium. This was mainly due to the prevention of leaf growth.

The proliferation of buds into meristemoid clusters commonly occurs in shaken or rotated liquid cultures (ZIV, 1991 and 1999), especially where cytokinins and / or growth retardants are used to control hyperhydricity (ZIV, 1992[a] and 1999). In the case of meristemoid formation in *C. moorei*, high cytokinin levels substituted for growth retardant effects in preventing leaf growth and in inducing meristemoid cluster formation (ILAN *et al.*, 1995; ZIV, 1995). This is not uncommon. For example, leaf growth of potato, *Nephrolepis*, *Philodendron*, *Gladiolus* and *Brodiaea* in liquid media, and the induction of meristemoid clusters were controlled by cytokinins and / or growth retardants (ZIV, 1991) e.g. kinetin and adenine sulphate (ZIV, 1999). In the mass propagation system for *Lilium auratum* in liquid shake culture (TAKAYAMA & MISAWA, 1983[b]), bulb scale extension was significantly reduced by kinetin with the result that their mean length was less than 5 mm at high concentrations. Their appearance was reportedly also abnormal. Cytokinins are known to enhance adventitious bud development in gelled media. Their effect is, however, more marked in liquid culture, possibly because plant growth regulators, like BA, are more effective in controlling the proliferation and regeneration potential of tissues in liquid culture as the cells are in direct contact with the medium (ZIV, 1995).

Thus cytokinins, at high concentrations, can be used to control hyperhydricity in liquid cultures by inducing nodular clusters with arrested leaf growth (ZIV, 1992[a] and 1992[b]; ZIV, 1995); and are just as effective as growth retardants.

FACTORS AFFECTING SHOOT AND BULBLET INDUCTION FROM MERISTEMOID CLUSTERS

Introduction

The development of meristemoids in liquid culture is usually followed by mechanical separation and inoculation onto hardening media for acclimatization and in preparation for transfer to the soil (ZIV, 1989; LILIEN-KIPNIS *et al.*, 1992). Alternatively, the meristematic or bud clusters may be used for further proliferation and biomass production in bioreactors (ZIV, 1997). Another possibility is to induce the development of storage organs as these reduce the problem of abnormal shoot morphogenesis (hyperhydricity) commonly associated with liquid cultures (ZIV, 1989). The differentiation and growth of storage organs, *in vitro*, further reduces the need for acclimatization (ILAN *et al.*, 1995) and so their development either in liquid media or after subculturing to the hardening stage is seen as an advantage for bioreactor technology (ZIV, 1997). Liquid media are sometimes preferred to agar or solidified media for storage organ formation, since they stimulate bulblet growth within a shorter time and lead to an increase in bulblet size and weight (ZIV, 1989; TAKAYAMA *et al.*, 1991). It is more common though for buds to be transferred to an agar-based medium for hardening (ZIV, 1989). The process is influenced by sucrose concentration, the balance of plant growth regulators, temperature and in some cases, the photoperiod and ammonium / nitrate ratio (ILAN *et al.*, 1995).

The differentiation of *Gladiolus* cormels occurred only if a cytokinin was present (GINZBURG & ZIV, 1973; cited by STEINITZ & LILIEN-KIPNIS, 1989). BA treatments, with an optimum dose response at 10^{-7} M BA, were identified as capable of stimulating corm development in *Gladiolus* plants (STEINITZ *et al.*, 1991). In combination with paclobutrazol, they produced larger corms, which suggests an interactive effect of BA and paclobutrazol (STEINITZ *et al.*, 1991). The effect may be established by first growing shoots on an agar medium containing BA and then transferring the shoots to a liquid shake culture medium supplied with paclobutrazol. Early and rapid *Gladiolus* corm development can also be promoted by applying cytokinins. These, too, are inhibitors of GA-biosynthesis (STEINITZ *et al.*, 1991). When supplied together, growth retardants can affect the level of cytokinins as well as ethylene (ZIV, 1990[a]). Other reports show that cormlet development *in vitro* is independent of exogenously supplied hormones (DANTU & BHOJWANI, 1987; cited by STEINITZ & LILIEN-KIPNIS, 1989).

Different levels of sucrose affect bulblet number and size in *Lilium* (TAKAYAMA, 1991). Whereas more bulblets were produced in cultures containing 3% sucrose, levels of 9% resulted in larger sized micro bulbs. Elevated sucrose concentrations (6%) also enhanced bulbing in *Ornithogalum dubium* (ZIV & LILIEN-KIPNIS, 1997[a] and 2000) and, at 9%, tuberization in potatoes (AKITA & TAKAYAMA, 1988). Supplementation of the liquid medium with high concentrations of sucrose may also condition *Gladiolus* shoots to earlier and rapid corm regeneration (STEINITZ *et al.*, 1991) and increase corm size (ZIV, 1990). The advantages are easier transplanting and storage (ZIV *et al.*, 1995).

Differentiation of *Lilium* bulbs occurred with greater frequency on charcoal-containing agar media rather than in shaken or static liquid cultures (TAKAYAMA & MISAWA, 1983[a]). Bulblet development in solidified media for *C. moorei* was also promoted by the inclusion of 5 g ℓ^{-1} activated charcoal.

Materials and methods

Shoot primordia and meristematic nodules were separated from the meristemoid or bud clusters initiated in liquid-shake MS medium containing 10 mg ℓ^{-1} BA. These were transferred to solid MS media as follows: (1) 10 mg ℓ^{-1} BA (control), (2) 1 mg ℓ^{-1} BA, (3) 6% sucrose and (4) 5 g ℓ^{-1} activated charcoal. All explants were placed in the light.

Results

Multiple shoots (2 - 10, sometimes more) developed from the meristemoid clusters in all treatments (Table 6.4 and Figure 6.3). These were hyperhydric (vitrified), especially in the media containing BA and sucrose. Bulblets only formed in response to lower (1 mg ℓ^{-1}) BA concentrations and supplementation by activated charcoal (Table 6.4 and Figure 6.3). On average, more than one bulblet developed from each meristemoid cluster when the medium contained activated charcoal. This was statistically significantly different to the result achieved on the medium supplemented with 1 mg ℓ^{-1} BA.

Table 6.4: The effect of BA, sucrose and charcoal on shoot growth and bulblet induction from meristemoid clusters (after 14 weeks)

Media supplements	Shoot elongation (%)	Bulblet induction (%)	Mean number of bulblets / explant
10 mg ℓ^{-1} BA (control)	100	-	-
1 mg ℓ^{-1} BA	68	24	0.32^{b}
6% sucrose	76	-	-
5 g ℓ^{-1} activated charcoal	28	64	1.12^{a}

Different letters show significant differences between treatments at the 5% level (ANOVA).

Discussion

The effect of placing *C. moorei* meristematic clusters on the same medium as that used for liquid-shake culture, but gelled instead of liquid, was to encourage shoot elongation. In other practices, meristematic clusters are transferred to solid media since this leads to enhanced organogenesis and bulblet development (ZIV, 1989; LILIEN-KIPNIS *et al.*, 1992; ZIV & LILIEN-KIPNIS, 2000). Meristems with green leaf primordia, for example, arose from morphogenic suspension cultures of garlic following transfer to an agar-solidified medium (NAGASAWA & FINER, 1988); bulb scales of *Lilium auratum* (TAKAYAMA & MISAWA, 1983[b]) vigorously developed bulblets and single buds or bud aggregates (clusters of 3-4 buds) of *Gladiolus* (ZIV, 1989; ZIV, 1990[a] and 1990[b]) formed cormlets. *In vitro* treatments for enlarging the bulbs of *Narcissus papyraceus* produced in liquid shake culture also takes place on solidified media containing 3% sucrose (BERGOÑÓN *et al.*, 1992).

No *C. moorei* bulblets were observed in hardening media containing BA at a concentration of 10 mgℓ^{-1}. But when the concentration was significantly reduced (1 mg ℓ^{-1} BA), bulblet induction was enhanced. The bulbing response of meristematic clusters on solid medium mirrors that achieved for twin-scales (as reported earlier). In the latter case, 100% bulblet regeneration was recorded for twin-scales placed on a modified MS medium containing 0.1 mg ℓ^{-1} BA. However, the potential for twin-scales to form bulblets decreased as the concentration of BA increased.

In the case of *Gladiolus*, BA was found to inhibit cormel formation, whether it was supplied in the presence or absence of PAC (STEINITZ & LILIEN-KIPNIS, 1989). Solid media containing various combinations of auxin (NAA, IBA) and cytokinin (BA, 2iP) were used to induce the regeneration of *Nerine* x mansellii plantlets from meristematic clusters that had proliferated in liquid culture. Apparently though, bulblet development was only sporadic. This

Figure 6.3: The effect of BA, sucrose and charcoal on shoot growth and bulblet induction from meristemoid clusters

1. Shoots - 10 mg ℓ^{-1} BA (control)
2. Bulblets - 1 mg ℓ^{-1} BA
3. Stunted shoots - 6% sucrose
4. Multiple bulblets - 5 g ℓ^{-1} activated charcoal

was attributed to the effects of PAC (LILIEN-KIPNIS *et al.*, 1992) which was thought to have inhibited morphogenesis. Later studies showed that bulblet formation could be enhanced by using auxin in the medium (ZIV *et al.*, 1995).

Lilium bulb scales produced bulblets when transferred to media without kinetin (TAKAYAMA & MISAWA, 1983[b]). In contrast, the plant growth regulators ABA, NAA and BA, when added to the hardening medium for *Gladiolus* micropropagation, ensured cormlet development as the buds proliferated (ZIV, 1989). The stimulating effects of cytokinin on corm formation were also observed for *Gladiolus* shoots grown in BA-containing media prior to cormlet production in liquid culture (STEINITZ *et al.*, 1991). An early application of cytokinin, thus ensured rapid corm regeneration in liquid cultures through its inhibition of GA-biosynthesis (STEINITZ *et al.*, 1991). An earlier report indicated that corm and cormel development in *Gladiolus* was paclobutrazol-dependent and that it probably mediated its effect by regulating endogenous levels of GA (STEINITZ & LILIEN-KIPNIS, 1989).

Although most of the meristematic clusters transferred to a medium containing 6% sucrose showed improved shoot elongation, these were noticeably hyperhydric. Furthermore, the shoots did not become swollen and produce bulblets, even though this had been recorded earlier for twin-scales supplied with the same concentration of sucrose.

Liquid media containing 30, 60 or 90 g ℓ^{-1} sucrose stimulated bulblet growth in *Lilium*. Higher concentrations (90 g ℓ^{-1}) in agar though, led to callus formation and the inhibition of bulblet development (TAKAYAMA & MISAWA, 1983[a]). However, the authors reported (1983[b]) that both the frequency and rate of bulblet regeneration for *Lilium auratum* was higher for bulb scales originating from bulblets on agar media containing 90 g ℓ^{-1} sucrose than those cultivated in liquid media.

Bud clusters of *Ornithogalum dubium*, when transferred from liquid or bioreactor cultures, developed bulblets in the presence of elevated agar and sucrose (6%) concentrations (ZIV & LILIEN-KIPNIS, 1997[a] and 2000). Aggregates of *Nerine* buds developed bulblets upon transfer to solid media containing 6% sucrose (ZIV, 1990[a]) or 6% sucrose in combination with low concentrations of auxin (LILIEN-KIPNIS *et al.*, 1992; ZIV *et al.*, 1995). *Gladiolus* cormlets developed in response to growth retardants, so long as there was sucrose in the medium (ZIV, 1989). Alternatively, storage organs that develop in liquid culture can be transferred to half-strength MS hardening medium with 6% sucrose for maturation e.g. *Gladiolus* sp. (ZIV, 1991). This concentration of sucrose was found to produce larger cormlets than the control medium containing only 3% sucrose (ZIV, 1990[b]). *Gladiolus shoots* that proliferated in an airlift bioreactor system were later transferred to an elevated sucrose medium for the

induction of corms (ZIV, 1998). Earlier reports for *Gladiolus* corm formation (STEINITZ *et al.*, 1991) also mention the promotive effects of sucrose (60 g ℓ^{-1}) on precocious corm formation when supplied to the liquid medium. The response is similar to sucrose-induced tuberization in potatoes (STEINITZ *et al.*, 1991).

The highest percentage bulblet regeneration from meristematic clusters of *C. moorei* grown in liquid culture was recorded on solid medium containing 5 g ℓ^{-1} activated charcoal. Enhanced bulblet development, as a consequence of employing charcoal as a constituent of the medium during routine micropropagation practices for *C. moorei*, is reported and discussed earlier in this work. Briefly, activated charcoal promotes growth by: (1) establishing a darkened environment; (2) adsorbing inhibitory compounds; (3) adsorbing growth regulators that may limit growth and (4) releasing growth-promoting substances adsorbed by the charcoal (PAN & VAN STADEN, 1998).

Another benefit of charcoal is its ability to reduce hyperhydricity, as was the case in shoot tip cultures of onion (SCHLOUPT, 1994; cited by PAN & VAN STADEN, 1998). To some extent, it also alleviated the occurrence of hyperhydric shoots that developed from the meristematic clusters of *C. moorei*. Debergh *et al.* (1981; cited by PAN & VAN STADEN, 1998) used charcoal to adsorb metabolites produced through cytokinin catabolism in the belief that these were the cause of hyperhydricity. There is also the possibility that charcoal may mediate its effects through its affinity to alkaloids. Amaryllidaceae alkaloids are able to form complexes with divalent metal ions (GHOSAL *et al.*, 1984). They are also biologically active and have been reported to promote root growth (GHOSAL *et al.*, 1984). Such activity may be effected by modulating the transport of metal ions across membranes (GHOSAL *et al.*, 1984).

CONCLUSIONS

Cultures of meristematic clusters of *C. moorei* proliferated in an agitated liquid suspension system containing BA. The cytokinin, at high concentrations, induced meristemoid cluster formation much like growth retardants (ZIV, 1992 and 1995); thereby controlling hyperhydricity. This served to corroborate the findings of Ziv (1992 and 1995). Shoot morphogenesis, followed by the formation of bulblets, was observed when the clusters were placed on a solid medium supplied with activated charcoal. Bulblet formation occurred also in the liquid medium when cytokinins were omitted.

Meristematic clusters have great potential for micropropagation in liquid cultures (ZIV *et al.*, 1994). The benefits include: "mechanical homogenization, separation and uniform propagule sizing, automated subculture and transfer, controlled shoot hyperhydricity in liquid media,

(and) high rates of proliferation and regeneration" (ZIV, 1999). Provided that they are manipulated appropriately, they can be induced to undergo organogenesis or somatic embryogenesis (ZIV *et al.*, 1994) and so form propagules ready for planting out. The use of liquid cultures for the *in vitro* propagation of ornamentals has been suggested as "the most likely means for facilitating automation and scaling up of plant production with concomitant saving in manual labour and significant reduction in production costs" (LILIEN-KIPNIS *et al.*, 1992). This has proved beneficial for the multiplication of *Crinum moorei* bulbs *in vitro* since by adopting a liquid-shake culture system, multiplication rates could be increased. For example, three "half" *in vitro* grown bulblets, when cultured in a liquid-shake medium, produced approximately 2.85 grams of regenerative material in the form of meristemoids. This material could be divided further to give 25 explants of 0.113 g each. Placing a single propagule on solid medium containing activated charcoal, on average, produced 1.8 bulblets. Effectively, this meant that the multiplication of the propagules in liquid-shake culture was approximately twice as productive as bulblet division, which normally takes place on solid medium. The time taken for bulblets to develop was also shortened. Whereas 7 shoots developed in 27 weeks following multiplication on solid medium, 15 bulblets were initiated in just 23 weeks in liquid-shake culture. The next step in scaling-up this process would be the employment of bioreactors.

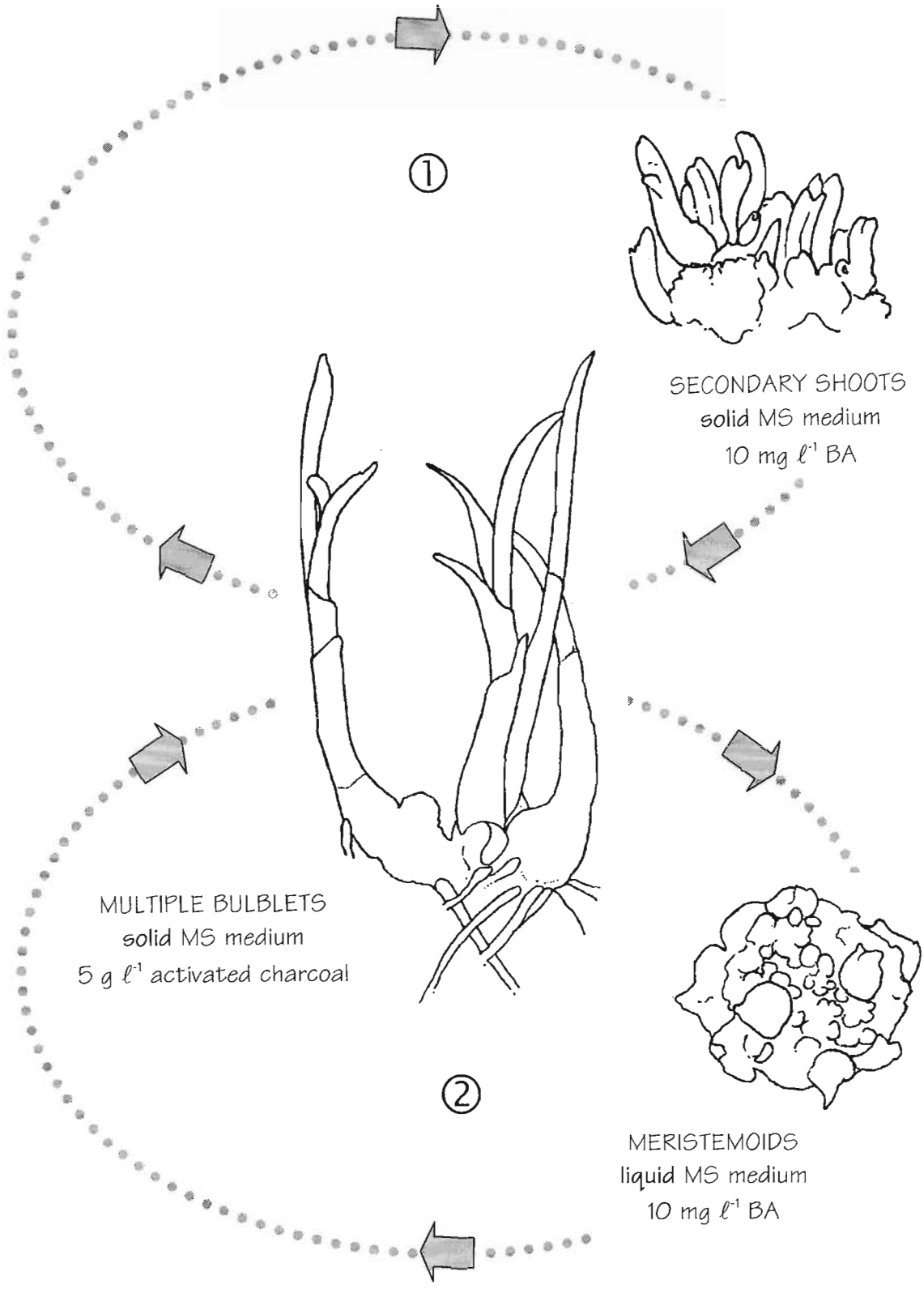


Figure 6.4: A summary of procedures used to multiply bulblets of *C. moorei*

1. Solid multiplication medium yields 7 shoots in 27 weeks (for each 'half' bulblet)
2. Liquid-shake multiplication medium yields 15 bulblets in 23 weeks (for each 'half' bulblet)

Chapter 7
SOMATIC EMBRYOGENESIS

INTRODUCTION

Somatic embryos and plant propagation

Somatic embryogenesis, as opposed to the maturation of zygotic embryos excised from seeds, is yet another technique of propagating plants *in vitro* (ZIV, 1997). Together with organogenesis, these represent the two major morphogenetic pathways for plant regeneration (ZIV *et al.*, 1994).

Single somatic cells are used to form embryos. These can be derived from such diverse cells as the epidermis of hypocotyls, vascular parenchyma in petioles, parenchyma in the storage tissues of roots and young inflorescences or even unfertilised gametic cells (AMMIRATO, 1983; MERKLE *et al.*, 1990). The resulting bipolar embryos are characterised by having a root, shoot and cotyledons and in this manner closely resemble zygotic embryos (AMMIRATO, 1983). They are in no way connected to the maternal tissue as they have a closed radicular end (TERZI & LOSCHIAVO, 1990). This is in contrast to organogenesis, which involves the regeneration of unipolar meristems which are usually multicellular in origin and connected by way of a procambial strand to the vascular tissues of the original explant (TERZI & LOSCHIAVO, 1990). The somatic embryos either develop into a complete plant or, as in the case of geophytes, directly into a storage organ (ZIV, 1997).

The advantages of somatic embryogenesis

Since somatic embryos are produced from single cells and remain genetically stable, somatic embryogenesis is accepted as having the greatest potential for producing large numbers of plants (MUJIB *et al.*, 1996; ETIENNE-BARRY *et al.*, 1999); especially elite plants which must be clonally propagated. Apart from the high-volume multiplication of embryonic propagules, the rate of plantlet conversion from embryos is reported to be equally as high e.g. in *Nerine* this was 10 - 50 times greater than that achieved with meristematic clusters (ZIV *et al.*, 1994). Many consider this to be the most commercially attractive application of somatic embryogenesis (MERKLE *et al.*, 1990) seeing that micropropagation in most cases is characterised by low multiplication rates.

Somatic embryos may be suspended in liquid medium and grown under controlled conditions (PREIL *et al.*, 1988). Shaking causes the embryonic tissue to divide frequently into smaller units (GUDE & DIJKEMA, 1997) and so liquid cultures are amenable to embryonic tissue proliferation (ETIENNE-BARRY *et al.*, 1999). As the embryos mature they can be harvested from the proliferating culture and converted to seedlings for transplanting or synthetic seeds for delivery to the grower (MERKLE *et al.*, 1990). Some workers consider this to be the most effective way of mass propagating plants "since it allows experimenters to grow essentially unlimited numbers of somatic embryos at low cost from bioreactor cultures" (LEVIN *et al.*, 1988), that is, provided plants are able to form somatic embryos in suspension culture and express low somaclonal variability (PREIL *et al.*, 1988). This was cited as the most practical means of mass propagating Nerines at a reasonable price (LILIEN-KIPNIS *et al.*, 1992). Scaling up in bioreactors has also been reported for several other species capable of regenerating somatic embryos (ZIV *et al.*, 1994). Somatic embryos are amenable to mechanical handling and so facilitate automation (AMMIRATO, 1983; MERKLE *et al.*, 1990).

The benefits of using somatic embryos over other forms of propagules is that they can be easily separated from the mother tissues (if necessary) and plated onto agar for further development without having to isolate and root individual plantlets as is the case for axillary and adventitious shoots (PREIL *et al.*, 1988; MERKLE *et al.*, 1990). This is because they are already bipolar in nature; a condition that is effectively achieved in one step (AMMIRATO, 1983). They have all the necessary "machinery" to become a whole new plant and are thus complete propagules in themselves (MERKLE *et al.*, 1990). It is also possible to sow somatic embryos directly in the field for plantlet conversion, bypassing the expensive stages of *in vitro* germination and acclimatization, which makes somatic embryogenesis a more cost effective means of plant production (ETIENNE-BARRY *et al.*, 1999). Furthermore, it is relatively easy to manipulate the different developmental stages (ZIV *et al.*, 1995). Somatic embryogenesis thus provides a convenient way of regenerating plantlets with concomitant savings in labour (GUDE & DIJKEMA, 1997).

Embryogenic material is ideal for genetic transformation, perhaps even more so in monocotyledons where embryogenic callus has proved to be the best target tissue for transformation (LIN *et al.*, 2000). As the establishment of an efficient regeneration system is regarded as a prerequisite for genetic modification, somatic embryogenesis has important implications for modern plant breeding research. Most breeders take advantage of the ability of single cells to regenerate plantlets as it means that cells with desired traits can be selectively multiplied (REMOTTI & LÖFFLER, 1995) and exploited for *in vitro* selection experiments (REMOTTI, 1995). For geophytes this is especially important in improving plant genotypes for the horticultural market (LIN *et al.*, 2000).

Another prospect for somatic embryos is their use in synthesizing certain metabolites where it remains impossible in undifferentiated tissues (AMMIRATO, 1983). The other alternatives are long-term storage through dormancy, artificial seeds (produced by the mechanical encapsulation of the embryos), cold storage or cryopreservation (AMMIRATO, 1983).

Somatic embryogenesis in ornamentals and bulbs

Propagation through somatic embryogenesis in geophytes has been achieved even in some of the most recalcitrant species (ZIV, 1997) e.g. *Tulipa* (GUDE & DIJKEMA, 1997; CUSTERS *et al.*, 1997; cited by ZIV, 1997), *Iris* (REUTHER, 1977; RADOJEVIC & SUBOTIC, 1992; cited by LILIEN-KIPNIS *et al.*, 1994), *Gladiolus* (KAMO *et al.*, 1990; cited by LILIEN-KIPNIS *et al.*, 1994; REMOTTI & LÖFFLER, 1995), *Freesia* (BACH, 1992; WANG *et al.*, 1994), *Hemerocallis* (KRIKORIAN & KANN, 1981; SMITH & KRIKORIAN, 1991; cited by GUDE & DIJKEMA, 1997) and *Nerine* (LILIEN-KIPNIS *et al.*, 1994; ZIV *et al.*, 1994). This route has also been used for plantlet regeneration of *Urginea indica* (JHA et al., 1991) and *Ranunculus asiaticus* (BERUTO & DEBERGH, 1992). By manipulating growth regulators and environmental factors, it has been possible to induce direct storage organ formation (ZIV, 1997).

Somatic embryogenesis in the Amaryllidaceae

Somatic embryogenesis has only been achieved for *Narcissus confusus* (SELLÉS *et al.*, 1999) and *Nerine* x *mansellii* (LILIEN-KIPNIS *et al.*, 1992 and 1994; ZIV *et al.*, 1995); two important ornamentals within the Amaryllidaceae. In the case of *Narcissus*, it was with a view to comparing the alkaloid content of galanthamine at different stages of morphogenesis.

Callus induction from mature embryos of *Narcissus* took place on solid medium containing either 2,4-D (2 or 4 mg ℓ^{-1}) or picloram (4 mg ℓ^{-1}) (SELLÉS *et al.*, 1999). When the callus was transferred to a high (10 mg ℓ^{-1}) auxin-containing medium, callus that had been initiated in the picloram treatments became embryogenic. Different callus strains were highly variable in their ability to produce shoots. Media containing 1 mg ℓ^{-1} BA gave the best organogenesis results with a high percentage of the calli converting to shoot clumps (SELLÉS *et al.*, 1999). Bulblets developed 1 - 2 months later on a medium supplemented with both BA (5 mg ℓ^{-1}) and 2,4-D (1 mg ℓ^{-1}). Both 2,4-D and BA were used to supplement the medium for callus induction in *Nerine* inflorescence explants (LILIEN-KIPNIS *et al.*, 1992). The callus-like tissue, interspersed with nodular regions, proliferated in a liquid medium to which had been added NAA, BA and paclobutrazol (PAC). The induction of proembryonic masses (green distinct protuberances) from the peripheral tissue of compact but friable meristematic clusters of *Nerine* was only possible when PAC, which had been included in the proliferation medium,

was removed, since it inhibited morphogenesis. Their subsequent development was enhanced with iP (LILIEN-KIPNIS *et al.*, 1992). Germination, however, took place in the absence of plant growth regulators in a liquid medium. The plantlets developed rooted bulblets upon transfer to semi-solid medium with 6% sucrose and 0.1 - 0.3 µM NAA.

Somatic embryogenesis in *Crinum*

There is only one report for the successful induction and germination of somatic embryos in the genus *Crinum* and that is for *Crinum asiaticum* (MUJIB *et al.*, 1996). Even here, while it was relatively easy to initiate callus from bulb scales and flower buds, only half the explants exhibited embryogenic potential and still fewer germinated.

Somatic embryogenesis: stages

Distinct stages are recognised in the development and maturation of somatic embryos: induction, early growth, embryo maturation and germination to form plantlets (LILIEN-KIPNIS *et al.*, 1994). These usually necessitate changes to the basal medium. For example, auxins and cytokinins are used to initiate and maintain callus formation; high concentrations of 2,4-D or other auxins are required to induce early embryo development and for proliferation while reducing or omitting the auxin leads to embryo maturation and growth into plantlets (AMMIRATO, 1983; LILIEN-KIPNIS *et al.*, 1994). Two different pathways of embryogenesis have been observed. In *Gladiolus* these were referred to as primary and secondary embryogenesis (REMOTTI, 1995) and were to a large extent influenced by the hormone composition of the medium.

AIMS

Somatic embryogenesis was investigated as an alternative pathway for the multiplication of *C. moorei*, since it lends itself to liquid or bioreactor culture. The primary aim was simply to assess the general feasibility of initiating and using somatic embryogenesis for micropropagating *Crinum moorei*, since this has not been attempted or reported elsewhere. This may help create a better understanding of factors affecting embryogenesis within the Amaryllidaceae where, in most cases, the seeds are recalcitrant.

CALLUS AND EMBRYO INDUCTION

Introduction

The induction of somatic embryos

Several factors are known to affect the embryogenic determination of tissues. Among the most important are the genotype of the starting material, origin of the explant and plant growth regulator composition of the medium (TERZI & LOSCHIAVO, 1990).

Although zygotic embryos are mostly used to induce callus that is embryogenic there are also reports where vegetative tissues such as axillary buds (LIN *et al.*, 2000), bulb scales, rhizomes, inflorescence stalks (LILIEN-KIPNIS *et al.*, 1992) and corms (LIN *et al.*, 2000) have proved successful amongst the bulbous monocotyledons (REMOTTI & LÖFFLER, 1995; LIN *et al.*, 2000); but these are few in number. *In vitro* grown material is sometimes used as well e.g. slices of *Gladiolus* cormels (REMOTTI & LÖFFLER, 1995) and the basal meristem of *in vitro* plantlets (KAMO *et al.*, 1990; cited by REMOTTI & LÖFFLER, 1995). Certain explants appear to be more amenable to callus production than others. This is especially so for monocotyledons. Usually it is the meristematic tissues that respond more readily (AMMIRATO, 1983) e.g. embryos, basal meristems and shoot tips (REMOTTI & LÖFFLER, 1995) and in one other case even seeds (MII *et al.*, 1994; cited by REMOTTI & LÖFFLER, 1995).

According to Lin *et al.* (2000), there are two types of callus produced in monocotyledons: compact and friable. The latter may be used to initiate suspension cultures. Generally the morphotype is determined by the type of culture medium used (LIN *et al.*, 2000) with 2,4-D directing the formation of friable callus and NAA compact callus in *Gladiolus* (KAMO *et al.*, 1990; cited by LIN *et al.*, 2000). The response of other tissues to plant growth regulators seems to be dependent on the species or plant part used. Highly embryogenic callus may require only a simple medium without plant growth regulators for initiation (GEORGE, 1993). But for differentiated explants from which unorganised callus is produced, the cells in the surrounding explant must be disrupted and / or high levels of the auxin 2,4-D used (MERKLE *et al.*,1990). In theory, it is therefore possible for any given cell to undergo embryogenesis, provided that it is given the appropriate stimuli and conditions (AMMIRATO, 1983). In almost all cases auxin or auxin-like compounds were found to be essential for callus induction and embryo induction (AMMIRATO, 1983). Cytokinins either enhanced the response or caused the tissues to respond in quite a different way (LIN *et al.*, 2000).

Although several auxins have been used to induce somatic embryos, including the natural auxin IAA and synthetic ones like NAA, 2,4-D is the auxin of popular choice (TERZI & LOSCHIAVO, 1990). This is because more embryogenic callus is invariably produced on media containing 2,4-D (REMOTTI & LÖFFLER, 1995). The effectiveness of the auxin is also related to its ability to maintain callus proliferation (REMOTTI & LÖFFLER, 1995). Auxins affect differentiated cells by inducing dedifferentiation. They also promote the formation of embryo primordia. Such seemingly contradictory responses may be related to the different sensitivities of tissues to auxins at different developmental stages. Frequently an interaction between the genotype and medium is found (REMOTTI & LÖFFLER, 1995). The promotive effect of auxins on morphogenesis is thought to occur because polar transport of the auxin is effectively blocked when concentrations are sufficiently high; resulting in increased intracellular levels of the hormone (TERZI & LOSCHIAVO, 1990).

In response to treatment with auxins, aggregations of compact cells arise which continue to divide in an asymmetric way. Clearly it is the high concentrations of auxins that cause dedifferentiation and elicit totipotency; one of the most critical steps in inducing somatic embryos. Collectively the cells, in clumps, are called proembryogenic masses or embryogenic clusters (TERZI & LOSCHIAVO, 1990).

Materials and methods

Twin-scales were placed on MS medium containing the following combinations and concentrations of 2,4-D and BA: 0:0, 0:1, 0:2, 0:4, 1:0, 1:1, 1:2, 1:4, 2:0, 2:1, 2:2, 2:4, 4:0, 4:1, 4:2 and 4:4 mg ℓ^{-1} (after LILIEN-KIPNIS *et al.*, 1992 and SELLÉS *et al.*, 1999). The explants were grown in the light at 25°C. Growth was monitored over 32 weeks. The percentage explants that produced embryogenic callus for each treatment (represented by 20 replicates) was recorded.

Results

Nodular and embryogenic-looking callus was observed in a range of treatments where 2,4-D and BA were both present (Figure 7.1). On its own, 2,4-D initiated callus production at a concentration of 2 mg ℓ^{-1}. This was also the best concentration to use in combination with BA as in all cases it gave the highest frequency of callus initiation. Overall, 2 mg ℓ^{-1} of both 2,4-D and BA resulted in the highest number (77%) of explants producing embryogenic callus. This result was, statistically, significantly different to the initiation response achieved on the medium containing 1 mg ℓ^{-1} of both 2,4-D and BA as well as on media containing only 2 mg ℓ^{-1} 2,4-D. The embryogenic callus appeared to originate from the active meristem at the base of the scales.

There were differences in the appearance of the callus. Distinct nodules of smooth yellow callus were embedded in translucent crystalline callus, while in other instances the callus resembled pro-embryogenic white clusters (Figure 7.2). The embryos occurred either individually or, in most cases, as clusters. Their smooth appearance suggested the presence of a protoderm. Later on some of the globular stage embryos enlarged and developed indentations where possible shoot apical meristems were forming.

Discussion

Explant effects

Like *C. asiaticum* (MUJIB *et al.*, 1996), embryogenic callus was successfully induced using explants from the bulb of *C. moorei*. Previously in this work, however, difficulties had been encountered in establishing callus from bulb scale tissue, so this result was perhaps somewhat unexpected. In other investigations though, bulb explants of *Urginea indica* yielded callus (JHA *et al.*, 1991) as did the bulb scales of tulip (GUDE & DIJKEMA, 1997). Corm producing species like *Freesia hybrida* produced somatic embryos when meristems were used as starting material (BACH, 1992). Callus capable of regenerating plantlets via somatic embryos was also produced from *in vivo* grown cormels of *Gladiolus* x *grandiflorus* Hort. (REMOTTI & LÖFFLER, 1995; REMOTTI, 1995). Explants other than those from storage organs have been used as well. For *Freesia hybrida* and *Narcissus*, somatic embryos have been initiated from zygotic or mature embryos (BACH, 1992; SELLÉS *et al.*, 1999) while immature ovary tissue was used in preference to other tissues for callus induction in *Hemerocallis* (KRIKORIAN & KANN, 1981). Inflorescence explants were used for *Nerine* (LILIEN-KIPNIS *et al.*, 1992), floral buds for *Ranunculus* (BERUTO & DEBERGH, 1992) and stem explants for tulip (GUDE & DIJKEMA, 1997) and *Alstroemeria* (LIN *et al.*, 2000). Juvenile material was preferred for inducing callus for somatic embryogenesis in *Cyclamen persicum* (KIVIHARJU *et al.*, 1992) and included anthers, ovaries and zygotic embryos. The examples cited above illustrate the diversity of explants that are capable of producing somatic embryos; the only prerequisite perhaps being that the material is juvenile or meristematic.

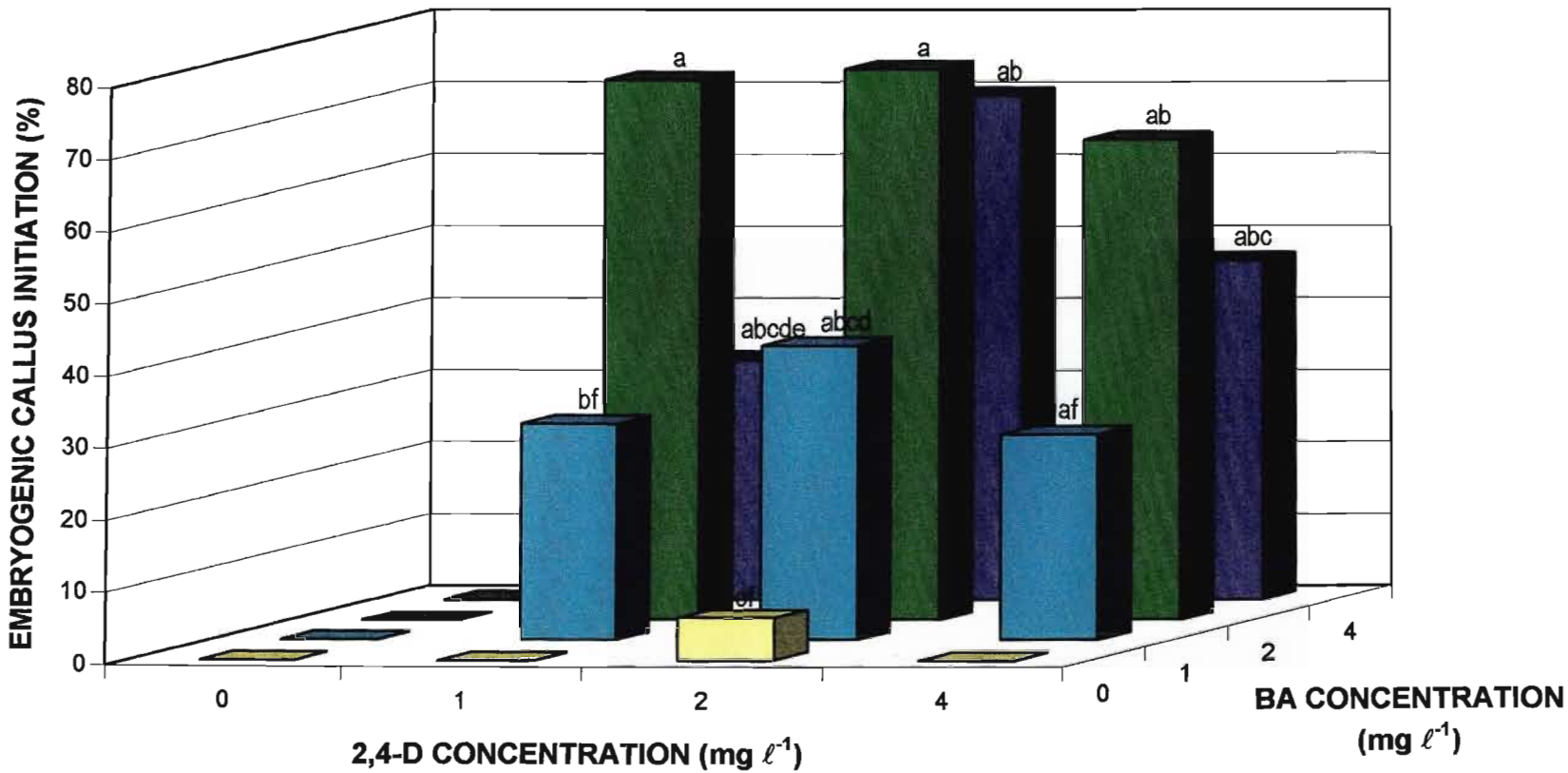


Figure 7.1: The effect of 2,4-D and BA on the initiation of embryogenic callus from twin-scale explants (after 32 weeks)
Different letters show significant differences between treatments at the 5% level (ANOVA)

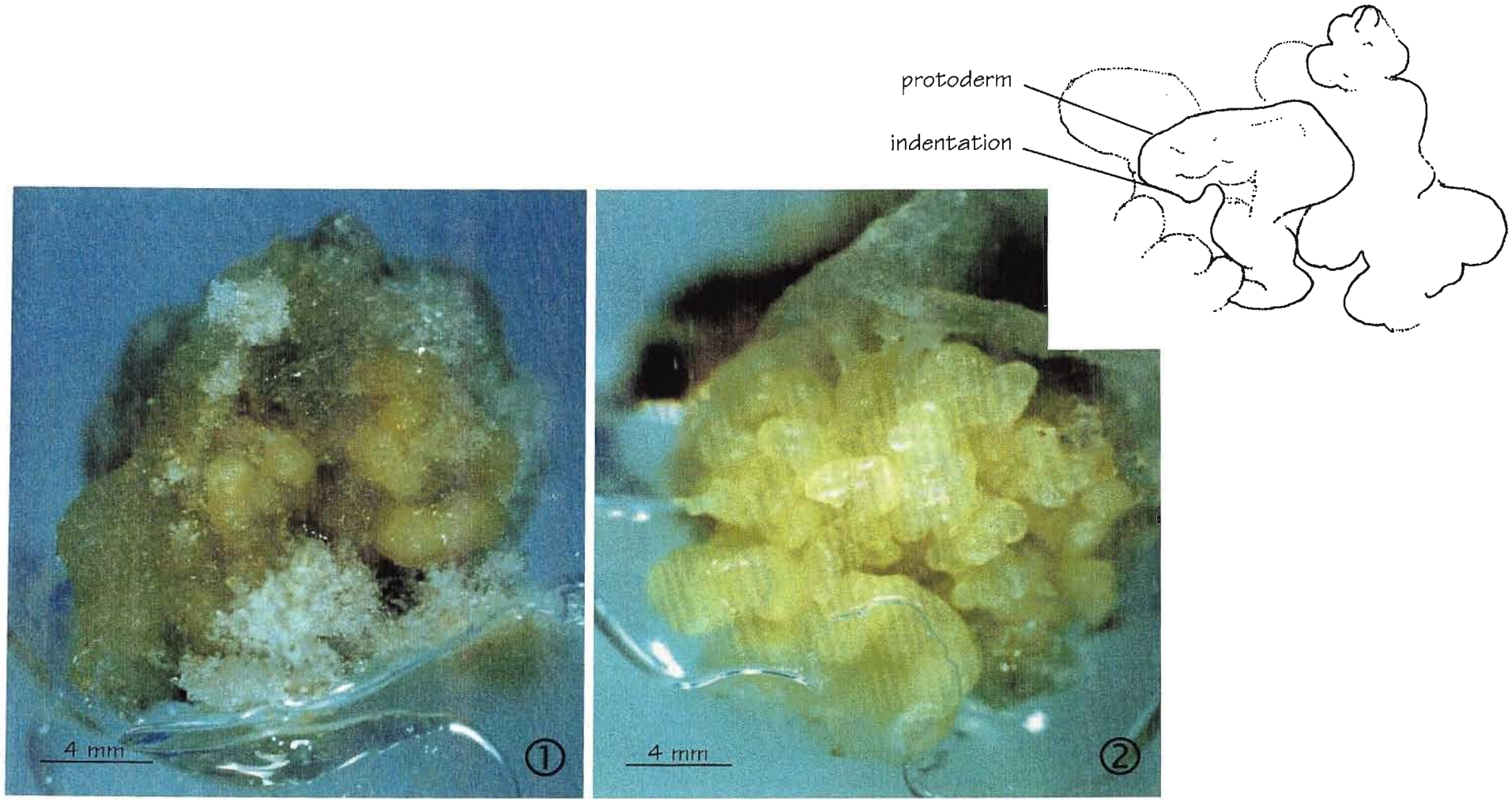


Figure 7.2: Embryogenic callus induction on MS medium containing 2,4-D and BA

1. Nodules of smooth yellow callus embedded in translucent crystalline callus (2 mg ℓ^{-1} 2,4-D + 4 mg ℓ^{-1} BA)
2. Pro-embryogenic white clusters (4 mg ℓ^{-1} 2,4-D + 2 mg ℓ^{-1} BA)

The induction of the embryogenic state: the role of auxins and cytokinins

In almost all cases auxins are required to induce somatic embryogenesis. For *C. moorei* the auxin 2,4-D could be used alone. The frequency of callus initiation, however, improved when 2,4-D was combined with the cytokinin BA in the induction medium.

The auxin that is used most often for initiating and maintaining embryogenic callus is 2,4-D. Callus from floral buds of *Ranunculus asiaticus*, for example, could be initiated and kept on a medium containing 2,4-D at a concentration of 1.6 mg ℓ^{-1} (BERUTO & DEBERGH, 1992). And although a range of media types were successful in inducing callus formation from cormels of *Gladiolus* x *grandiflorus* Hort. (REMOTTI & LÖFFLER, 1995), only 2,4-D was able to maintain growth since on NAA the callus degenerated. In a comparative study, an increasing callus response was effective with 2,4-D and not NAA (STEFANIAK, 1994; cited by REMOTTI & LÖFFLER, 1995). The auxins 2,4-D and picloram (0.5 - 50 µM) had the same effect in producing undifferentiated as well as "meristematic" callus from tulip tissues (GUDE & DIJKEMA, 1997). Higher concentrations (5 or 50 µM) produced more callus. The auxins 2,4-D and picloram were also used for callus formation in *Narcissus* (SELLÉS *et al.*, 1999). It is however possible that somatic embryogenesis can be induced using auxins other than 2,4-D. An earlier report for *Freesia*, for example, maintained that somatic embryogenesis from flower parts could be achieved using NAA (WANG *et al.*, 1990; cited by BACH, 1992). Where suspension cultures are used in propagating *Gladiolus*, NAA rather than 2,4-D was more effective for callus induction (KAMO, 1994; cited by REMOTTI & LÖFFLER, 1995). As an alternative, picloram has been suggested as a more effective inducer of embryogenesis in monocotyledons than NAA and 2,4-D (BACH, 1992).

The successful initiation of somatic embryogenesis in bulbous ornamentals can, in most instances, be attributed to the use of both auxins and cytokinins during the inductive phase. For example, auxins alone or in combination with cytokinins were successful in obtaining somatic embryos in *Freesia hybrida* (BACH, 1992) while hormone-free media or media containing only cytokinins never induced embryogenesis. Furthermore, both IAA and BA were used to initiate somatic embryos directly from young inflorescence stems of *Freesia refracta* (WANG *et al.*, 1994). For *Nerine*, 2,4-D in combination with BA resulted in callus production (LILIEN-KIPNIS *et al.*, 1992). The callus-like tissue, interspersed with nodular regions, proliferated in a liquid medium to which had been added NAA, BA and paclobutrazol (PAC). Proembryonic masses arose when paclobutrazol was removed from the proliferation medium. Where stem explants were used as starting material in tulip, BA was essential for both callus initiation and meristematic nodule formation (GUDE & DIJKEMA, 1997). Although 2,4-D (0.5 - 4.0 mg ℓ^{-1}) could be used on its own for callus initiation in *Alstroemeria* stem segments (LIN

et al., 2000), combined with BA at concentrations between 0 - 2 mg ℓ^{-1}, it yielded friable callus that was necessary for somatic embryo development. Proembryos (yellow structures measuring 1 mm in diameter and enclosed by a protoderm) also formed directly from the compact callus, depending on the combination of hormones used (LIN *et al.*, 2000). Coconut milk or kinetin (1 mg ℓ^{-1}) were equally effective in yielding callus from the bulb explants of *Urginea indica* provided that the medium was also supplemented with 2,4-D (2 mg ℓ^{-1}) (JHA *et al.*, 1991). Callus could be maintained for a year on medium containing only 2,4-D, but tetraploid varieties needed 2 mg ℓ^{-1} kinetin for callus induction as well as maintenance (JHA *et al.*, 1991). The auxin 2,4-D and coconut milk (CM) were necessary for initiating somatic embryogenesis in *Cyclamen persicum* (KIVIHARJU *et al.*, 1992). Occasionally though, embryos developed in the induction medium without CM, provided that the ammonium ion level was increased. In the case of *Crinum asiaticum*, 2,4-D and BA were used alone or BA combined with NAA to induce callus formation either from bulb scales or flower buds. Without changing the composition of the plant growth regulators, somatic embryos developed spontaneously (MUJIB *et al.*, 1996). Cell suspensions of Daylily are capable of giving rise to embryonic structures when the level of 2,4-D is reduced and the larger masses of tissue in suspension are subsequently transferred to a modified White's or Schenk and Hildebrandt medium (KRIKORIAN & KANN, 1981). But for embryogenesis in *Narcissus*, it was necessary to transfer the picloram-treated callus to a medium with a higher auxin concentration (SELLÉS *et al.*, 1999).

It is perhaps possible that differentiated tissues require cytokinins to be present, that is, in combination with auxins, in order to induce embryogenesis (AMMIRATO, 1983). For although the majority of studies have used only auxins (2,4-D and NAA), this has been to induce embryogenesis in immature embryonic explants (MERKLE *et al.*, 1990). Genotype, tissue type and developmental stage of the explant tissue are thus important factors in determining the response of the tissues to hormonal supplements. While BA alone can induce somatic embryogenesis in pre-embryogenic determined cells (PEDCs), in the hypocotyl region of immature embryos, auxins effect a response in a much wider range of tissues and at different stages of development (MERKLE *et al.*, 1990). On their own they have the potential to give rise to induced embryogenic determined cells (IEDCs) even from nonembryogenic tissue (MERKLE *et al.*, 1990).

Direct and indirect embryogenesis

In this study, proembryonic masses were visible in the primary culture. This phenomenon has been reported elsewhere (AMMIRATO, 1983). In the latter case it was assumed that the callus was in reality a collection of small globular proembryos that were prevented from maturing because of the auxin in the medium. Thus it appears that there are two pathways for embryogenesis from somatic cells; firstly direct embryogenesis, where the embryos develop directly from explant cells with an organised structure (WANG *et al.*, 1994) and without callus proliferation and secondly, indirect embryogenesis which involves a certain amount of cell proliferation along with the formation of recognisable embryonic structures (AMMIRATO, 1983; MERKLE *et al.*, 1990). In other words, cells may be either pre-embryogenic determined cells (PEDCs) or induced embryogenic determined cells (IEDCs). This depends on whether they are epigenetically embryonic at explanting or whether they are induced during somatic embryogenesis (MERKLE *et al.*, 1990). "The direct formation of embryos in culture is an event that needs the ordered expression of stimuli provided by endogenous cellular factors or applied culture conditions" (REMOTTI, 1995). Furthermore, there appear to be differences in the patterns of initial cell divisions between direct and indirect embryogenesis (WANG *et al.*, 1994). There has been some uncertainty as to the origin of somatic embryos, but most authors agree that somatic embryogenesis occurs from single cells, in which case genetic variation is avoided (WANG *et al.*, 1994). Embryogenesis, in the early stages, is manifested in proembryonic groups of cells developing a thick, cutinised wall which effectively separates them from surrounding cells (MERKLE *et al.*, 1990).

MATURATION OF EMBRYOS AND THE DEVELOPMENT OF GERMINABILITY

Introduction

The next stage in the process of somatic embryogenesis involves the initiation of bipolar differentiation and the formation of cotyledons and root and shoot apices from proembryonic masses. These must be present (and functional) in order for germination to occur. The embryos should easily convert to plantlets to facilitate transfer to the soil (MERKLE *et al.*, 1990). Many physical and media-related factors are known to affect morphogenesis i.e. amino acids, nitrogen, carbohydrates (in their capacity to increase the osmotic concentration of the medium), exogenous hormones (e.g. ABA), evolved gases and light (AMMIRATO, 1987). From early studies it was shown that a reduction in the auxin concentration used to initiate embryogenesis, or its complete removal, was critical for embryo maturation and germination (AMMIRATO, 1983). For if present at high concentrations, auxins can inhibit development as

well as the outgrowth of the shoot meristem in proembryos (MERKLE *et al.*, 1990). The removal of auxin also prevents recurrent embryogenesis (MERKLE *et al.*, 1990).

Materials and methods

To determine whether the embryogenic callus could be induced to form shoots, a range of BA concentrations and BA and NAA in combination, were tested. Since the proembryos could easily be separated into individual units and separated from the underlying callus surface, these were transferred to MS media containing the following combinations and concentrations of plant growth regulators: (1) 0 mg ℓ^{-1} BA + 0 mg ℓ^{-1} NAA, (2) 0.1 mg ℓ^{-1} BA, (3) 1 mg ℓ^{-1} BA and (4) 1 mg ℓ^{-1} BA + 0.1 mg ℓ^{-1} NAA. The cultures were placed in the light and grown at a temperature of 25°C. Embryo maturation was assessed over a period of 35 weeks. The numbers of embryos that germinated within a sample size of 25, for each treatment, were recorded.

Results

Morphological differences were observed in the development of the proembryos. While for some there was no change in their appearance after 35 weeks, others became swollen and were either white or green, or swollen with a distinct pointed outgrowth. These were bulblet-like in appearance (Figure 7.3). The best germination response (22%) occurred on the medium supplemented with 1 mg ℓ^{-1} BA (Table 7.1). In most cases the embryonic shoot matured into a bulblet no different from the bulblets produced by adventitious budding (Figure 7.4).

Table 7.1: The effect of plant growth regulators on embryo maturation and germination (after 35 weeks)

BA + NAA (mg ℓ^{-1})	**Growth response** Embryo germination (%)
0 : 0	13
0.1	0
1	22
1 : 0.1	13

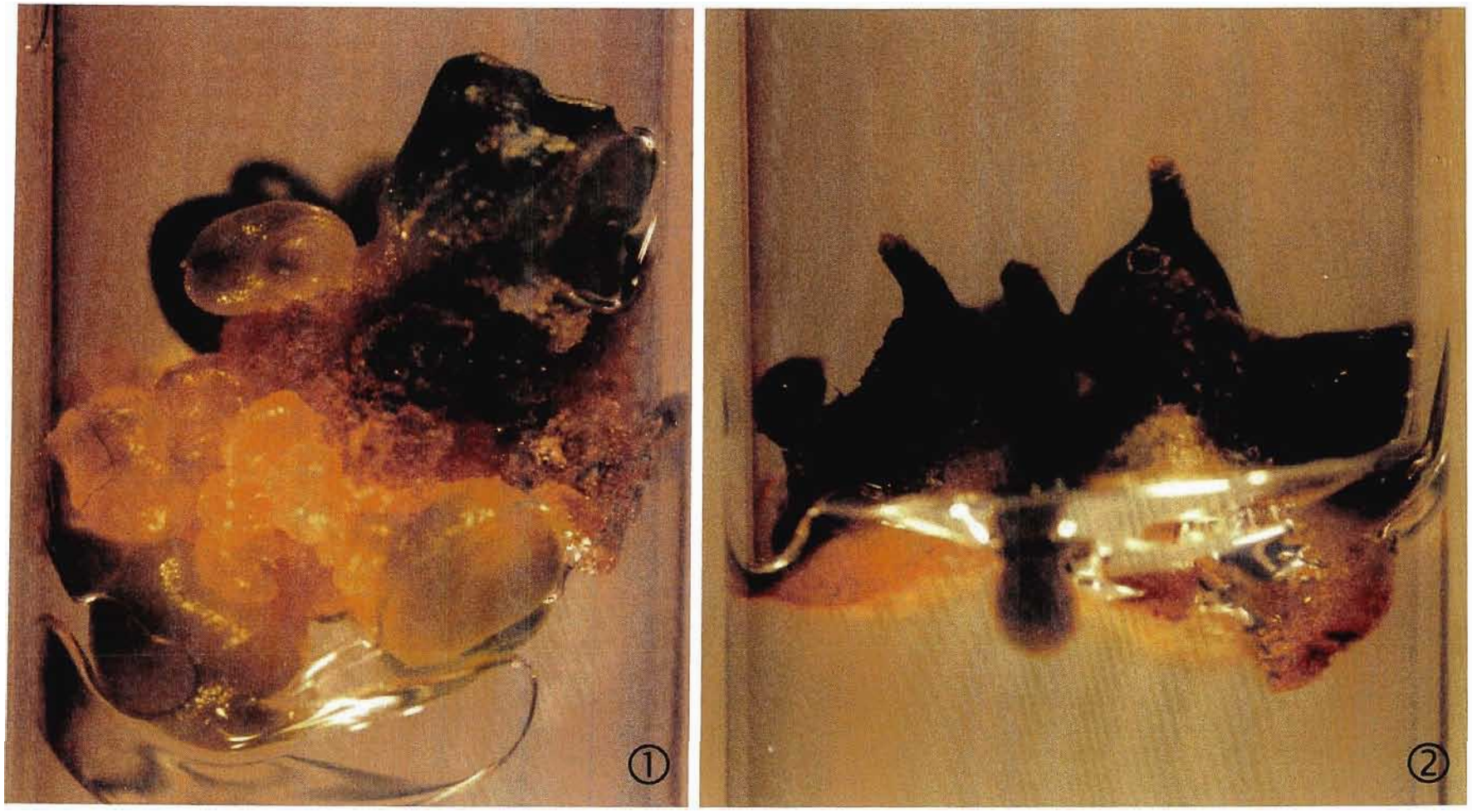


Figure 7.3: The effect of plant growth regulators on embryo maturation and germination

1. Embryos swollen (0.1 mg ℓ^{-1} BA)
2. Pseudobulblets (1 mg ℓ^{-1} BA + 0.1 mg ℓ^{-1} NAA)

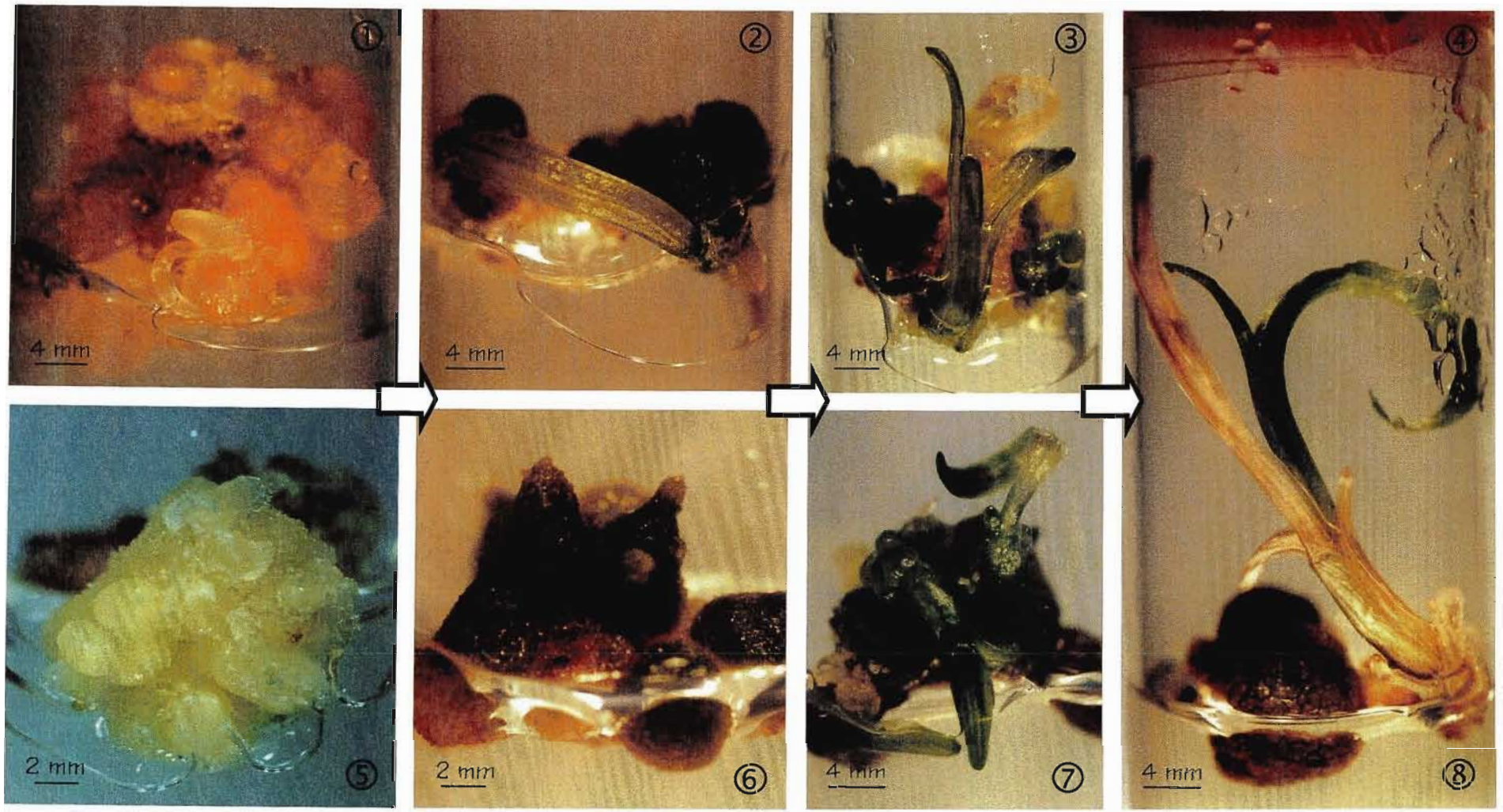


Figure 7.4: Embryo maturation and germination: a developmental sequence through two different pathways

1.	Embryogenic callus	2.	Shoot	3.	Shoot elongation	4.	Bulblet formation
5.	Embryogenic callus	6.	Pseudobulblets	7.	Shoot emergence	8.	Bulblet formation

Discussion

The effect of plant growth regulators

The conversion of embryos into plantlets usually proceeds upon removal of the auxin. Its omission from the medium is thus generally regarded to be the prerequisite for complete embryo maturation. To some extent this was also true for the germination of *C. moorei* somatic embryos and the embryogenic cultures of *Freesia hybrida* (BACH, 1992) and the daylily cultivar Autumn Blaze (KRIKORIAN & KANN, 1981). In both *Freesia* and daylily, fertile plants regenerated when the level of auxin was drastically reduced or almost completely removed. Sometimes maturation proceeds spontaneously perhaps because 2,4-D is progressively consumed by the embryos (REMOTTI, 1995).

Substituting the auxin with a high cytokinin to auxin ratio results in an improved germination response for many species of bulbous ornamentals. Proembryogenic and early embryo development in *Nerine* was only possible on a medium with a low auxin to cytokinin ratio (LILIEN-KIPNIS *et al.*, 1994). In this case the authors noted a difference to other reports for embryo maturation in monocotyledonous species where there was a need for auxins at higher concentrations.

Alternatively, media containing only cytokinins, like BA, may achieve organogenesis as effectively as auxins and cytokinins in combination or result in accelerated maturation e.g. *Alstroemeria* (LIN *et al.*, 2000), *Freesia* (BACH, 1992) and *Narcissus* (SELLÉS et al., 1999). Plant regeneration from yellow compact callus of *Gladiolus* x *grandiflorus* Hort. took place on a range of media supplemented with zeatin and BA (REMOTTI & LÖFFLER, 1995). It was generally noted that increasing the dosage of zeatin led to a concomitant increase in plantlet regeneration (REMOTTI & LÖFFLER, 1995). A combination of zeatin and BA was better for plantlet regeneration than kinetin on its own (KAMO, 1994; cited by REMOTTI & LÖFFLER, 1995). Plantlets germinated from embryo-like structures on media with zeatin or BA (0.25 µM) in the case of "Peter Pears"; a cultivar of *Gladiolus* x *grandiflorus* (REMOTTI, 1995). The combination of cytokinins (BA and zeatin) and or reduced level (0.5 µM) of BA affected development i.e. embryo maturation stopped once the structures were spherical. Thus the number of regenerated plants per callus colony was strictly dependent on the applied cytokinin (REMOTTI, 1995). Although no plant growth regulators were required for the germination of *Nerine* embryos, the development of proembryonic masses (green distinct protuberances) was enhanced by 2iP after PAC had been removed from the medium since it was thought to inhibit morphogenesis (LILIEN-KIPNIS *et al.*, 1992).

Other reports have shown that the development of embryo germinability may not be dependent on hormones. An embryogenic cell culture of daylily, for example, was found to be capable of producing somatic embryos in hormone-free medium after a short maintenance period on hormones (SMITH & KRIKORIAN, 1991). The transition from preglobular stage to germinable embryos was facilitated by altering the pH to 5.8 and or placing the embryos on filter paper impregnated with activated charcoal (SMITH & KRIKORIAN, 1991). Plant regeneration from differentiating embryogenic cell suspensions of *Gladiolus* x *grandiflora* occurred when the embryo-like structures were placed on solidified hormone-free medium and allowed to dry (REMOTTI, 1995). Furthermore, no hormones were required for the embryogenic callus of *Urginea indica* to produce leafy shoots (JHA *et al.*, 1991) or for the germination of *Nerine* somatic embryos in liquid medium (LILIEN-KIPNIS *et al.*, 1994). The meristematic and nodular type of callus in *Tulipa*, which was thought to be in the early stages of somatic embryogenesis, could also easily regenerate plantlets when it was transferred to a hormone-free medium or a medium containing only low (0.5 µM) concentrations of 2,4-D or picloram (GUDE & DIJKEMA, 1997). Embryoid differentiation in callus from *Ranunculus asiaticus* also occurred when the callus was transferred to hormone-free medium or on media containing low auxin levels. This was dependent on the composition of the callus initiation medium (BERUTO & DEBERGH, 1992). All the somatic embryos developed into plantlets. Morphologically mature embryos were already observed in induction media for *Cyclamen persicum*. The presence of 2,4-D, therefore, did not prohibit embryo maturation as has been reported in other studies. Although it was not necessary for the medium to be altered, the absence of plant growth regulators was more conducive to embryo germination (KIVIHARJU *et al.*, 1992). In fact, the highest germination frequency was achieved in media lacking 2,4-D.

In several cases the production of bulblets may require an alteration to the basal medium used for embryo germination; either through the addition of different hormone combinations or increasing the concentration of sucrose. Bulbing in *Narcissus*, for example, took place when both BA and 2,4-D were included in the medium (SELLÉS *et al.*, 1999). No bulb formation in *Urginea indica* followed in media free of plant growth regulators although this was possible upon addition of NAA (0.01 - 0.05 mg ℓ^{-1}) and kinetin (0.05 mg ℓ^{-1}) (JHA *et al.*, 1991). *Nerine* plantlets, however, developed rooted bulblets upon transfer to semi-solid medium with 6% sucrose and 0.1 - 0.3 µM NAA. It may be that the high sucrose levels, in altering the osmotic potential of the medium, facilitated their development (LILIEN-KIPNIS *et al.*, 1994).

The balance of hormones is thus the most important factor in directing the course of somatic embryogenesis. In several cases cytokinins such as kinetin have proved useful in promoting embryo maturation, especially the development of cotyledons and their growth into plantlets (AMMIRATO, 1983).

The developmental anatomy of somatic embryos

It is generally accepted that somatic embryos develop superficially on callus and for this reason are easily detached from the surrounding cells (GEORGE, 1993). In monocotyledons, their development is different to that of dicotyledons. In short, the embryos of monocotyledons give rise to shoots from discrete globular bodies sometimes through stages that can be distinguished by the formation of a scutellar notch, scutellum and coleoptile (GEORGE, 1993). Smith and Krikorian (1991) refer to these stages as (1) the globular embryo stage (an indentation marks the site of the future stem apex), (2) the cotyledon initiation stage and (3) the plumule stage.

An anatomical and morphological investigation of somatic embryogenesis in *Tulipa* showed that the meristematic or nodular callus formed as a continuation of the vascular bundle in the bulb scale (GUDE & DIJKEMA, 1997). In *Freesia* where inflorescence explants were used to initiate callus, the somatic embryos grew out of single cells in the outer part of the vascular bundle sheath. The authors suggest that this may be related to higher levels of plant growth regulators and nutrients in the vascular tissues (WANG *et al.*, 1994). Where floral buds were used to initiate callus in *Ranunculus* (BERUTO & DEBERGH, 1992), embryoids developed either from cells at the surface of the callus or from cells lying deeper. Somatic embryos also arose from dark brown callus in *Cyclamen persicum* (KIVIHARJU *et al.*, 1992), individually or as clusters. Those of *Nerine*, however, developed from meristematic centres in the ground parenchyma tissue of the inflorescence-derived explants (LILIEN-KIPNIS *et al.*, 1994). These proembryos were initially limited to just a few cells. Eventually they became multicellular and elongated with a distinct cotyledon containing a procambial strand (LILIEN-KIPNIS *et al.*, 1994).

In most instances the embryos closely resemble those formed during zygotic embryo development. The embryos in *Tulipa*, for example, possessed a leaf-like cotyledon with a procambial strand, and at its base, a cavity where an additional meristem was located (GUDE & DIJKEMA, 1997). The somatic embryos in *Alstroemeria* were similar in structure, with a cotyledon, shoot apex and root primordium (LIN *et al.*, 2000). In the cotyledonary stages of development in *C. asiaticum*, the embryos were distinguished by a large furrow at the apex (MUJIB *et al.*, 1996). Embryos which arise directly from callus may possess a suspensor e.g. *Freesia refracta* (WANG et al., 1994) and in cases where embryogenesis is indirect, the parent tissue may substitute for the suspensor (WANG *et al.*, 1994).

Abnormalities

In this study some of the proembryogenic masses did not complete their development into somatic embryos or when transferred to the maturation medium developed abnormally. This is not unusual for callus which consists of globular bodies, possibly because conditions are not conducive for embryo development. The globular bodies increase in size (up to 4 mm in diameter) without germinating. These so-called pseudobulbils probably develop because polarity is not established when they reach the 16-32 cell stage (BUTTON & BOTHA, 1975; cited by GEORGE, 1993). Furthermore, somatic embryos exhibit a high degree of plasticity during early development (AMMIRATO, 1987; cited by LILIEN-KIPNIS *et al.*, 1994). Thus any slight changes to the medium composition are likely to have a profound effect on morphogenesis.

Poor germination response

Other studies have also reported low germination frequencies for somatic embryos. For example, in *C. asiaticum*, embryogenic callus that was initiated on bulb scales in the presence of 2,4-D, did not germinate at all or at very low frequencies (MUJIB *et al.*, 1996). To some extent, the problem could be overcome by using BA (4.4 µM) and NAA (2.68 µM) instead. And, whereas only 20% of *Gladiolus* embryos germinated directly on hormone-free solid medium, those placed on top of filter paper gave a 60% germination response and fewer neomorphs (REMOTTI, 1995). Liquid media have proved advantageous for somatic embryogenesis in other ornamental monocotyledons e.g. *Hemerocallis* and *Gladiolus* (BACH, 1992).

CONCLUSIONS

Embryo development in *C. moorei* was facilitated by removing 2,4-D from the culture medium or substituting this with a low concentration of NAA (0.1 mg ℓ^{-1}). The best maturation response was achieved on a medium supplemented with 1 mg ℓ^{-1} BA. Few however, went on to germinate with the result that the regeneration frequency was not significantly high. Somatic embryogenesis is thus not an efficient route for micropropagating *C. moorei* at present, because of the low frequencies of embryo production and germination and long culture times. Because this phase of the project was simply to assess the feasibility of somatic embryogenesis for *C. moorei*, it was not intended that each of the steps be optimised. The most critical stage is ensuring that sufficiently large numbers of the pro-embryonic structures go on to germinate for this proved to be inconsistent. Thus although both organogenic and embryogenic pathways are used for large-scale liquid cultures (ZIV, 1995), proliferation in liquid culture is currently a better proposition for this species.

However, because some of the conditions required for initiation and maturation were established, future research could focus on improving the rate of production of mature embryos that readily germinate. The use of liquid media in this respect may be one option.

Apart from using somatic embryogenesis as a micropropagation route, somatic embryos could be used to determine alkaloid production *in vitro* in plant tissues in varying stages of morphogenesis, since it has been shown that somatic embryos are sufficiently organised for alkaloids to accumulate. How tightly the synthesis of alkaloids is correlated with the differentiation of cells into embryos or bulblets, makes for an interesting developmental study.

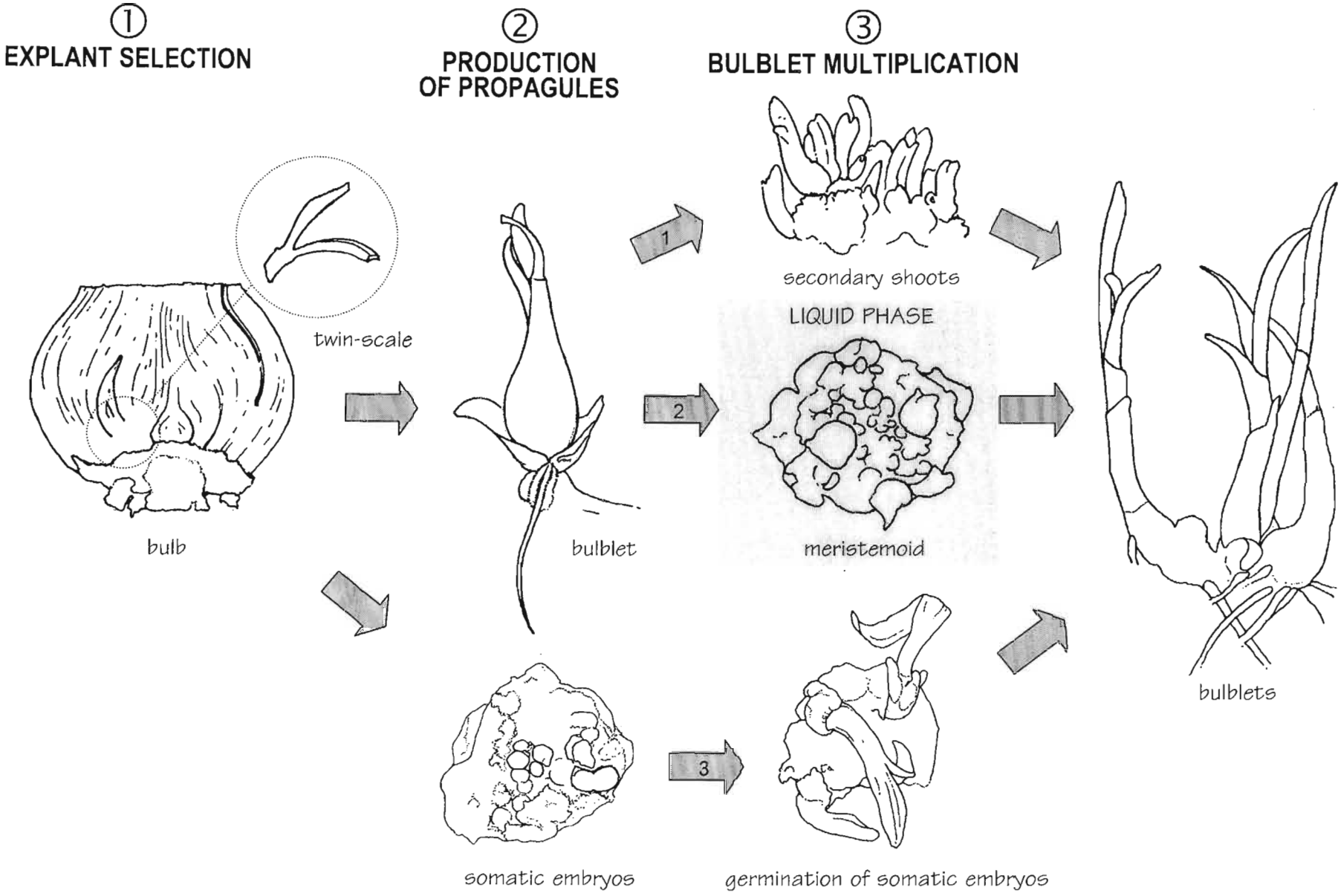


Figure 7.5: A summary of pathways, including somatic embryogenesis, for the production of *C. moorei* bulblets
1. Organogenesis 2. Organogenesis in liquid culture 3. Somatic embryogenesis

Chapter 8
THE PRODUCTION OF *CRINUM* ALKALOIDS *IN VITRO*

INTRODUCTION

"Plants are extraordinary chemical factories" (HUXLEY, 1984). They are thus an important source of organic compounds, many of which form the basis for development of medicinally important drugs because they are pharmaceutically active. Other plant-derived natural products are speciality chemicals used in insecticides, flavours, fragrances and dyes (VERPOORTE, 1998). These - secondary metabolites or compounds - are specialised substances that differ from plant to plant. That is, they are taxonomically restricted. They may be final products of metabolism or metabolic intermediates (GIULETTI & ERTOLA, 1999). Although they may not be essential for plant life, they play a major role in the plant's interaction with the environment (VERPOORTE, 1998).

Secondary products of economic importance

Secondary metabolism is characterised by a diversity of chemical structures (GIULETTI & ERTOLA, 1999). Three important groups of secondary compounds are the alkaloids, glycosides and saponins (HUXLEY, 1984).

Owing to the fact that there is increasing interest in the use of medicinal plants as phytomedicine preparations or herbal remedies (GIULETTI & ERTOLA, 1999), 400 of which are used commercially (HUXLEY, 1984), "plant compounds are big business" (HUXLEY, 1984). In 1980, world trade stood at $550 million (HUXLEY, 1984). An estimated 25% of the prescription drugs in the USA are derived from higher plants. In addition, synthetic analogues are based on prototype compounds isolated from plants (GIULIETTI & ERTOLA, 1999).

Sources of secondary metabolites derived from plants

Secondary metabolites of pharmaceutical importance are made available through: (1) isolation, either from wild plants or agronomic cultures, (2) chemical synthesis, (3) biotechnological processes such as cell cultures and genetically transformed hairy roots or (4) combined techniques (GIULUETTI & ERTOLA, 1999).

Increasingly, difficulties are encountered in securing supplies of medicinal plants. This is largely a result of the decline in resources because of human disturbance of the natural environment and ruthless exploitation, as well as increasing labour costs and technical difficulties associated with plant production (TABATA, 1977). The situation is even more serious in developing countries (GIULIETTI & ERTOLA, 1999) where the protection of plants has not been legislated or enforced and because a large percentage of the population depends on these plants for their primary health care needs. As the plant kingdom represents an important source of new compounds that may potentially be used in treating cancers, HIV and as contraceptives (GIULIETTI & ERTOLA, 1999), it is imperative that these resources be conserved.

In addition, many such compounds are present in low yields, since the biosynthesis and accumulation of the majority of secondary products are restricted to specialised cell types and to different phases of development (GIULIETTI & ERTOLA, 1999). Furthermore, they may be difficult to isolate. These present problems when large quantities of biologically active compounds are required (KUTNEY, 1995).

Few of the 30 or so therapeutically used alkaloids have been chemically synthesized (ANDERSON *et al.*, 1986). This is because many are complex structures, some even having chiral centres (GIULIETTI & ERTOLA, 1999). Plants are thus the only effective and economically viable means of synthesis (GIULIETTI & ERTOLA, 1999).

Natural products in plant tissue and cell suspension cultures

In the 1970's, scientists recognised that it was possible to produce useful and commercially important secondary products in cell cultures (YAMADA & HASHIMOTO, 1990). Since it is feasible to cultivate plant cells on a large scale, it is possible that products could be produced for about 400 - 1500 US$ per kg if productivities were in the order of 0.3 to 3 g ℓ^{-1} (VERPOORTE, 1998). This approximates the prices of several natural products used as drugs produced by more conventional means but is economically more profitable for some compounds e.g. taxol and vinblastine (VERPOORTE, 1998).

Cell cultures may be employed for the synthesis of: (1) established products; (2) products from plants which are difficult to grow or establish; (3) novel products (GIULIETTI & ERTOLA, 1999) and (4) compounds via biotransformation (FOWLER, 1983), as plant cell cultures are a source of key enzymes necessary for catalysing specific biosynthetic steps (GIULIETTI & ERTOLA, 1999).

For the most part, research efforts aim to increase the production of secondary compounds and reduce their price (BAJAJ *et al.*, 1988).

Advantages of using an in vitro system

There are advantages to producing compounds in culture. Where plants are endangered (PHILLIPSON, 1990), not domesticated, difficult to cultivate in field conditions or exhibit slow growth (GIULIETTI & ERTOLA, 1999), cell, tissue or organ cultures provide alternatives to the whole plant for molecule hunters. A reduction in land use or changing land use patterns (DICOSMO & MISAWA, 1995) may also limit plant cultivation. This may mean that plants need to be micropropagated to provide sufficient material for extraction purposes. Furthermore, there is independence from environmental factors such as climate, pests, geography, soil and seasonal variation and freedom from socio-political problems (GIULIETTI & ERTOLA, 1999) such as political interference or instability (FOWLER, 1983) when tissue-cultured plants are used. *In vitro*, production may be defined and optimised because plants are grown under standardised conditions; guaranteeing constant and stable supplies (STABA, 1980; GIULIETTI & ERTOLA, 1999). It is also possible to select high-yielding strains (DICOSMO & MISAWA, 1995) and achieve more consistent quality and yield of the product (STÖCKIGT *et al.*, 1995). Where undifferentiated cells are used, problems associated with translocation, permeability and segregation of metabolic pools are minimised such that there is better precursor incorporation (STABA, 1980). The ease with which products are recovered from cell cultures, in particular, favours extraction and purification especially when the compounds are proteins (GIULIETTI & ERTOLA, 1999). Another advantage is that cell growth is quicker in bioreactors (ALFERMANN & PETERSEN, 1995). The elucidation of the biosynthetic pathway; isolation of enzymes involved as biocatalysts in the pathway (STÖCKIGT *et al.*, 1995) and the relationship between organogenesis and secondary metabolite production (BAJAJ *et al.*, 1988) are made possible through the use of *in vitro* systems. Other benefits include the establishment of genetic improvement programmes (GIULIETTI & ERTOLA, 1999) and, in some cases, the production of novel compounds (PHILLIPSON, 1990).

Review: Secondary metabolite production in vitro

About 100 000 plant secondary metabolites are known (VERPOORTE, 1998) and of these, a range has been observed in plant cell cultures (FOWLER, 1983). Compounds belonging to more than 50 different categories of compounds have so far been recovered from cell cultures (BAJAJ *et al.*, 1988). These are mainly phenylpropanoids, alkaloids (FOWLER, 1983), terpenoids and quinones (STÖCKIGT *et al.*, 1995).

Alkaloids are of economic importance and therefore of interest to biotechnology (WINK, 1987). "Of the pharmacologically active principles found in plants, alkaloids are arguably the most important group" (ANDERSON *et al.*, 1986). Many are used therapeutically since they cover a broad spectrum of pharmacological effects (ANDERSON *et al.*, 1986). Families known to be rich in alkaloids e.g. Amaryllidaceae, were the first to be studied in order to determine whether the alkaloids were produced *in vitro* and at what levels (STABA, 1980). About 200 or more alkaloids have been produced by plant cell cultures (NICKELL, 1980; PHILLIPSON, 1990). These cover almost all the main alkaloid types i.e. indoles, isoquinolines, quinolines, quinolizidines, tropanes, acridones and purines (ANDERSON *et al.*, 1986). "A great deal of interest has focussed on the indoles and isoquinolines which represent the two main groups of plant alkaloids" (ANDERSON *et al.*, 1986). Mostly, this is because isoquinolines constitute a major group of plant-derived pharmaceuticals (ZENK *et al.*, 1988). Of the 80 plant-derived drugs widely used in western medicine, 16% are isoquinolines (ZENK *et al.*,1988). "Together with the indole alkaloids, they are the most important class of therapeutic agents obtained from plants" (ZENK *et al.*,1988).

Types of cultures for secondary metabolite synthesis

Biotechnological strategies employed in the synthesis of secondary metabolites of pharmaceutical interest include: (1) micropropagation, to provide uniform and pathogen-free material and (2) plant cell, tissue and organ culture for *de novo* synthesis and / or biotransformation (GIULIETTI & ERTOLA, 1999).

There are currently two types of *in vitro* cultures used for the production of secondary metabolites. These are dedifferentiated cultures, including callus and suspension cultures or differentiated cultures, such as transformed roots and shoots (GIULIETTI & ERTOLA, 1999). Suspension cultures and transformed root cultures are the methods used most extensively (GIULIETTI & ERTOLA, 1999).

Over 30 cell culture systems have been shown to be better producers of secondary metabolites than the intact plant (WINK, 1987). Usually, though, plant cells accumulate secondary metabolites in small amounts; much less than the intact plant (GIULIETTI & ERTOLA, 1999). Low product yields may be attributed to repressed enzyme activity (DICOSMO & MISAWA, 1995). Usually, it is the absence of differentiated cells that is accompanied by a decrease or loss in secondary metabolite synthesis (GIULIETTI & ERTOLA, 1999). Alkaloids are especially difficult to produce in cell cultures, or so it is generally thought. This may be due to a metabolic block at a specific step in the biosynthetic

pathway (TABATA, 1977). As a result, commercial production is not viable (GIULIETTI & ERTOLA, 1999).

Although cell cultures have been used for the production of secondary metabolites, there has been little commercial success. The disadvantages associated with using dedifferentiated cultures, namely: that it is difficult to obtain callus; that cell growth is slow and heterogenous (GIULIETTI & ERTOLA, 1999); and that synthesis only takes place in differentiated cells (FOWLER, 1983), means that differentiated cultures are often a better option. Thus, increasingly the trend has been toward using organ cultures. This is because it is impossible, in many cases, to uncouple secondary metabolite synthesis from morphological development (FOWLER, 1983). The cells in organogenic cultures are "under much tighter metabolic control than cells in suspension cultures because they remain organised as structures" (BURBIDGE, 1994). The result is increased productivity in such systems (BURBIDGE, 1994). Differentiated cells have secondary metabolite profiles that in many ways are similar to the intact plant (GIULIETTI & ERTOLA, 1999). Since synthesis may be restricted to a specific organ and is influenced by the specific stage of plant development (WINK, 1987), hairy root cultures are commonly employed. Shoot cultures may also prove useful as a source of secondary compounds (YAMADA & HASHIMOTO, 1990). Differentiated cultures may either be transformed or non-transformed (GIULIETTI & ERTOLA, 1999).

Production of Amaryllidaceae alkaloids *in vitro*

A large number of plants belonging to the Amaryllidaceae have been characterised chemically. They contain a group of isoquinoline alkaloids - the Amaryllidaceae alkaloids - which are sufficiently distinct from the chemical components of the Liliaceae to give them independent family status (RAFFAUF, 1996).

About 45 isoquinolines have been detected in plant cell cultures (ANDERSON *et al.*, 1986), including the more important opium alkaloids - papaverine, thebaine, codeine and morphine - produced by *Papaver somniferum* and *P. bracteatum*; berberine, which is synthesized by *Coptis japonica* and sanguinarine, found in *Macleaya* plants (STABA, 1980). "Some of the major successes in the production of secondary metabolites from plant cell cultures, both in terms of highest yielding cultures and biosynthetic studies, have been obtained with isoquinoline alkaloids" (ANDERSON *et al.*, 1986). The elucidation of the berberine biosynthetic pathway, at the enzyme level, has, for example, been achieved through the application of plant cell cultures (ANDERSON *et al.*, 1986). "Their medicinal value, chemical complexity and structural diversity make them an ideal group of natural products for the study of biosynthesis and biotechnological production" (ZENK *et al.*, 1988). In addition, quantities

sufficient to meet commercial demands are not easily obtained as they are present in limited supply or else the plant materials are expensive (ZENK *et al.*, 1988).

The following alkaloids are reported in cultures of members of the Amaryllidaceae (Table 8.1).

Table 8.1: Amaryllidaceae alkaloids produced *in vitro*

Amaryllidaceae alkaloid	Plant species	Type of culture	Culture conditions	Reference
Galanthamine (morphine-like)	*Narcissus confusus*	callus, somatic embryos and shoot clumps		Sellés *et al.*, 1999
N-formylnorgalanthamine		shoot clumps	high (9%) sucrose	Sellés *et al.*, 1997[b]
		buds		Sellés *et al.*, 1997[a]
Haemanthamine		shoot clumps (alkaloids released into the medium)	light and / or dark (inhibition by precursor trans-cinnamic acid)	Bergoñón *et al.*, 1996
Tazettine				
Pancratistatin	*Hymenocallis littoralis*	callus and bulblets		Backhaus *et al.*, 1992
Unidentified alkaloid	*Hippeastrum vittatum*	callus		Suhadolnik, 1964

The pharmaceutical importance of galanthamine

Galanthamine, one of the chemotaxonomical markers in the Amaryllidaceae family (RAUWALD *et al.*, 1991) and among the 21 alkaloids reportedly found in *Crinum moorei*, has been prominent in the popular and scientific press (LEWIS, 1996 and 2000). It was discovered accidentally by a Bulgarian pharmacologist in wild Caucasian snowdrops in the early 1950's (GREENBLATT *et al.*, 1999). Plant extracts containing the compound were first used to treat pain and poliomyelitis (GREENBLATT *et al.*, 1999). Since then it has been tested for use in anesthesiology and for treating facial nerve paralysis, paralysis syndrome (LEWIS, 1999; GREENBLATT *et al.*, 1999), nervous diseases (HARBORNE & BAXTER, 1993; GREENBLATT *et al.*, 1999), schizophrenia and various dementia (GREENBLATT *et al.*, 1999; SRAMEK *et al.*, 2000), as well as Alzheimer's disease (GREENBLATT *et al.*, 1999). Galanthamine exhibits muscarinic (LEWIS, 1996) and anticholinesterase activity (GHOSAL *et al.*, 1985; MARTIN, 1987) in both the central and peripheral nervous systems (GORINOVA *et al.*, 1993).

Galanthamine acts in a similar manner to other Alzheimer's drugs by replenishing acetylcholine levels in brain areas lacking cholinergic neurones (EICHHORN *et al.*, 1998) to enhance cholinergic function (SELLÉS *et al.*, 1997[a]). This it does by binding to the active site of the brain enzyme acetylcholinesterase and in so doing, blocks acetylcholine breakdown (GREENBLATT *et al.*, 1999). Interestingly, inhibition of acetylcholinesterase is also the principle mode of action of many pesticides. Researchers at the Weizmann Institute of Science have therefore proposed that plant products, like galanthamine, might act in defence against insects or parasites. In addition, it stimulates pre- and postsynaptic nicotinic receptors which can, in turn, increase the release of neurotransmitters like acetylcholine and glutamate (SRAMEK *et al.*, 2000), thus directly stimulating neuronal function. The stimulation of nicotinic receptors is also suggested to protect against β-amyloid toxicity (SRAMEK *et al.*, 2000). In theory, the release of additional acetylcholine is of benefit to the patient, although its usefulness has yet to be clinically proven (SRAMEK *et al.*, 2000). Its dual mode of action (GREENBLATT *et al.*, 1999), coupled with the evidence that galanthamine has reduced side effects, make it a promising candidate for the treatment of Alzheimer's disease (GREENBLATT *et al.*, 1999) and for designing improved potency drugs. However, given that galanthamine, at present, does not offer advantages in efficacy, safety or convenience over related products, it is its stimulation of nicotinic receptors and potential benefits thereof, that offer exciting prospects for further clinical research (SRAMEK *et al.*, 2000).

In early clinical trials galanthamine stabilized patients' symptoms for up to one year (BONNER, 1995). More recently it has been shown to improve cognitive function and have a beneficial effect on behavioural symptoms, provided that the treatment is administered early (SRAMEK *et al.*, 2000). Improvement in daily living is also associated with its usage (SRAMEK *et al.*, 2000). As a consequence, the United States, EU and Switzerland have approved the use of galanthamine (marketed as the hydrobromide salt Reminyl®) for the symptomatic treatment of mild-to-moderate Alzheimer's disease (SRAMEK *et al.*, 2000) although this has, as yet, not been granted in Canada, Australia and South Africa (SRAMEK *et al.*, 2000).

Another noteworthy pharmacological action of galanthamine is its ability to amplify nerve-muscle transfer (GHOSAL *et al.*, 1985). This is achieved by reversing non depolarizing neuromuscular block and restoring synaptic transmission (MARTIN, 1987).

Clinical investigations of galanthamine have resulted in several patents. Commercial products such as Nivalin™ and Energix™, for the treatment of poliomyelitis and other neurological diseases, are available in Eastern countries (SELLÉS *et al.*, 1999). In Eastern Europe it is used as a reversal agent in anaesthetic practice (EICHHORN *et al.*, 1998). It is also known

to inhibit traumatic shock (MARTIN, 1987) and has been patented for use in the treatment of nicotine dependence (LEWIS, 1996; GREENBLATT *et al.*, 1999). It is a nicotinic activator and acts on both ganglionic and muscle receptors and on nicotinic receptors in the brain (GREENBLATT *et al.*, 1999). Furthermore, galanthamine acts as a mild analeptic; is as powerful an analgesic as morphine; reduces intraoccular pressure when applied as eye drops and is used to treat several neurological disorders (EICHHORN *et al.*, 1998).

Until 1996 (BERGOÑÓN *et al.*, 1996), *Leucojum aestivum* L. was the only source of galanthamine which could be extracted on an industrial scale (GORINOVA *et al.*, 1993) and Bulgaria was the sole producer (GORINOVA *et al.*, 1993); having to meet the increasing demand for galanthamine both locally and by other international markets (GORINOVA *et al.*, 1993) despite the fact that the plant's natural habitat was reportedly diminishing in size (GORINOVA *et al.*, 1993). Threats to naturally occurring populations and the fact that the synthesis of galanthamine on an industrial scale is not yet feasible (EICHHORN *et al.*, 1998), have, therefore, stimulated attempts to find better sources in new *Narcissus* cultivars (MORAES-CERDEIRA *et al.*, 1997; LEWIS, 2000) and to produce the compound in culture. Planting depth, density, bulb size and flower bud removal did not, however, affect galanthamine content (MORAES-CERDEIRA *et al.*, 1997; LEWIS, 2000). In tissue culture, yields are "still on the low side" (LEWIS, 1999).

Apart from using biotechnology as an approach to producing galanthamine, and other related Amaryllidaceae alkaloids, *in vitro* techniques could also be used to study the biosynthesis of Amaryllidaceae alkaloids. The injection of radioactive precursors into the whole plant has, on previous occasions, presented several problems (SUHADOLNIK, 1964). Callus or cell suspension cultures provide an alternative system for determining the effect of alkaloids on enzyme levels (SUHADOLNIK, 1964). In addition, it is possible that structurally different compounds may be produced in differentiated cultures.

AIMS

Although *Crinum* species are known to contain pharmacologically active Amaryllidaceae alkaloids, notably galanthamine, their production *in vitro* has not been investigated. This would be important for several reasons. Members of *Galanthus* and *Leucojum* are used for the industrial extraction of galanthamine (SELLÉS *et al.*, 1997[b]), but, because they are difficult to obtain, they are an expensive source for clinical studies and usage. Even though the alkaloids have been isolated from *Crinum* species, these too are at risk in the wild; legally protected; rare and exploited by both the herbal medicine and horticultural plant trade (SCOTT-SHAW, 1999). The problem is further exacerbated by the fact that the plants are

difficult to propagate using conventional means i.e. by seed and vegetative offsets. Alkaloid yields are, in most cases, low and restricted to a period of about 10 weeks during the pre- and post flowering stages (GHOSAL *et al.*, 1985). Alkaloid content thus changes depending on the stage of plant development (GORINOVA *et al.*, 1993). Some of the *Crinum* type alkaloids have been synthesized (LEWIS, 1998). The total organic synthesis of galanthamine has also been established but the "stereoselectivity of its reactions, and the low yields make this process economically unattractive" (SELLÉS *et al.*, 1997[b]). As an alternative, plantlets regenerated *in vitro* could be used for alkaloid extraction. Manipulating the physical and nutritional status of the culture environment can also lead to a better understanding of factors likely to improve product yields. These are reported to be low in the only established system for galanthamine production: the shoot-clump cultures and regenerated plantlets of *Narcissus confusus* (SELLÉS *et al.*, 1997 and 1999).

The aims of this study were twofold. Firstly, to determine whether bulblets of *Crinum moorei*, produced *in vitro*, were capable of synthesizing alkaloids, and secondly, to establish whether their production could be increased by manipulating the culture environment. The presence of alkaloids in the culture medium was also investigated, since this has important implications for product recovery.

EXTRACTION, IDENTIFICATION AND QUANTIFICATION OF *CRINUM* ALKALOIDS

Materials and methods

Plant material

Bulblets (one to two years old) grown *in vitro* were used for alkaloid analysis. The bulblets and MS medium were analysed separately.

Drying and extraction

Following a method developed by Elgorashi (2000), the bulblets and media were dried at 55°C (50 days). The bulblets were then ground to a fine powder and weighed. Both the bulblets and media were extracted in dilute acid by adding 5 mℓ 0.05N HCℓ and shaking (150 rpm) at 40°C for 2½ hours. The solution was centrifuged (3600 rpm) for 5 minutes before adding 1 mℓ 0.3N NaOH and 4 mℓ chloroform to 3 mℓ of the extract. After centrifuging (4500 rpm) for 5 minutes, the chloroform layer containing the extract was filtered through anhydrous sodium sulphate. This was then air-dried. After adding 100 µℓ methanol (MeOH) to the dried

residue, 1 µℓ of the sample was injected into the gas chromatograph (GC) for analysis. For each sample, two injections were made and the mean concentration calculated.

The Varian 3300 gas chromatograph was equipped with FID and NPD detectors and a DB-5 capillary column (30 m x 0.32 mm i.d. x 0.25 mm film thickness; J&W Scientific, CA) and was linked to a Hewlett Packard 3395 Integrator. Nitrogen was the carrier gas with a head pressure of 40 KPa. Oven temperatures were set initially at 220°C, for 1½minutes, and then increased to 270°C at a rate of 3°C per minute. The injector and detector temperatures were 270°C and 300°C respectively.

Identification and quantification

To identify the alkaloids, the retention time was compared with that of a known sample. The amount of each alkaloid present in the sample was calculated by converting the area under each peak to a concentration value using regression plots defined for each alkaloid (ELGORASHI, 2000). The final concentration is expressed as mg 100 g^{-1} DW for both the bulblets and medium.

Results

Alkaloids present in the bulblets and medium

Nine alkaloids were identified in the bulblets of *C. moorei*: cherylline, crinamidine, crinine, epibuphanisine, lycorine, powelline, undulatine, 1-epideacetylbowdensine and 3-*O*-acetylhamayne. These represent three classes of Amaryllidaceae alkaloids (after MANSKE, 1975): lycorine, crinine and cherylline type alkaloids. Several other alkaloids were also present in the plant material tested (Figure 8.1), although it was not possible, at the time, to positively identify these. Three of the alkaloids present in the bulblets were also released into the medium, namely: crinine, powelline and undulatine (Table 8.2).

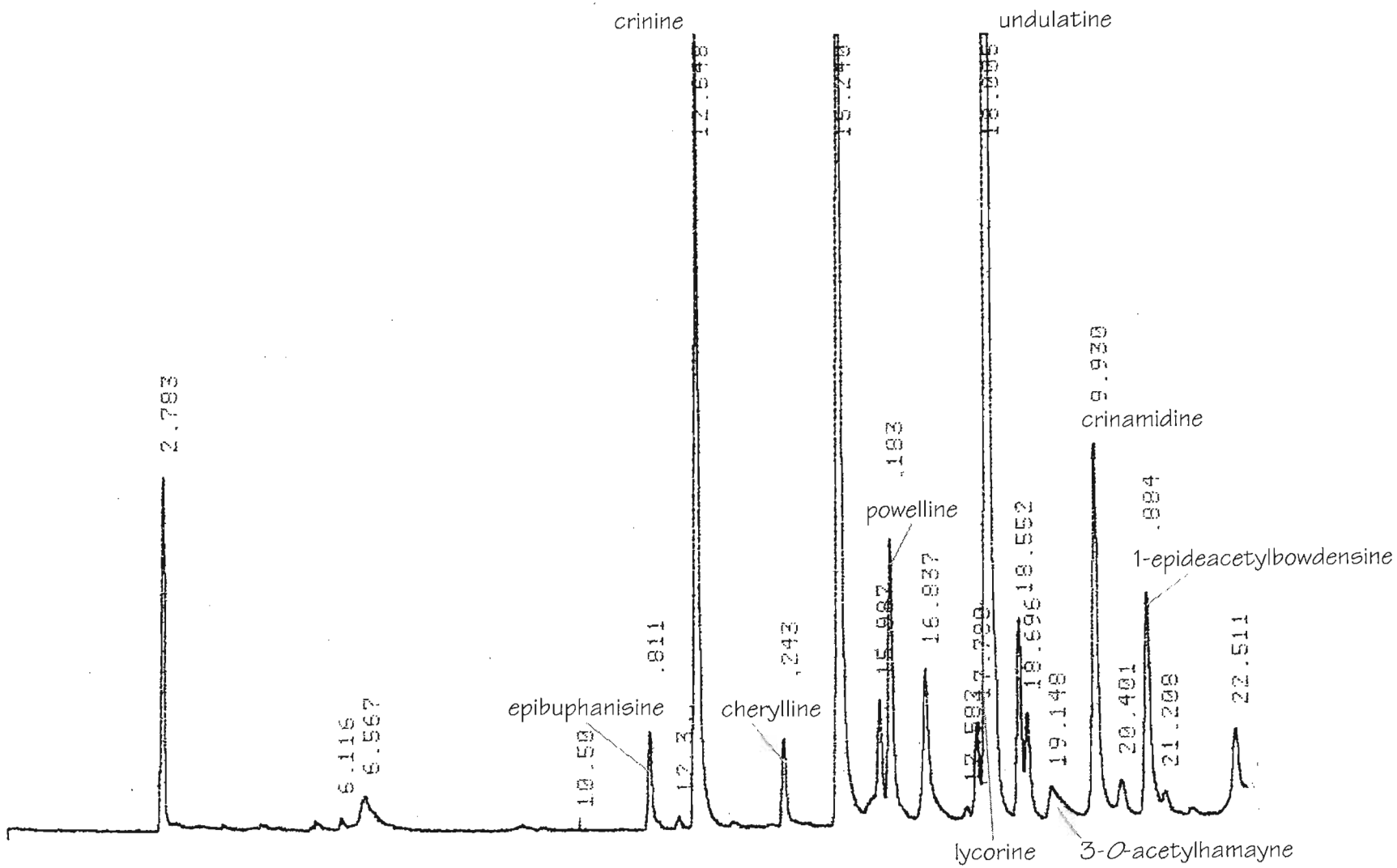


Figure 8.1: Alkaloids extracted from the *in vitro* grown bulblets of *C. moorei* and identified using gas chromatography

Table 8.2: Types and quantities of *Crinum* alkaloids present in the bulblets and medium

Crinum alkaloid	Bulblets of *Crinum moorei* (mg 100 g^{-1} dry weight)	Medium (mg 100 g^{-1} dry weight)
crinamidine	135.35	-
1-epideacetylbowdensine	32.79	-
undulatine	31.41	0.10
powelline	28.60	0.59
3-*O*-acetylhamayne	27.82	-
crinine	27.43	3.34
lycorine	5.21	-
epibuphanisine	3.05	-
cherylline	1.98	-

Discussion

Validity of methods

Using the method devised by Elgorashi (2000), it was possible, with slight modification, to achieve good separation of the alkaloids and to detect these even in small amounts of plant material (0.03 g - 0.15 g). This is an advantage in screening *in vitro* material.

Comparison of alkaloid yield from whole plants and plant tissue cultures

Nine alkaloids were detected in the bulblets of *C. moorei*. Except for 3-*O*-acetylhamayne, which is reported to occur in this species for the first time (Table 8.3), the alkaloids are all present in plants collected from the wild and represent 43% of the total number of alkaloids isolated from this species (ELGORASHI *et al.*, 2001[a]). Crinamidine was the most abundant alkaloid in the bulblets of *C. moorei*. This alkaloid also gave the highest yields for mature plants in the "wild" (ELGORASHI, 2000).

Table 8.3: Crinum alkaloids in "wild" and *in vitro Crinum moorei* bulbs

Alkaloid	Present in "wild" *Crinum moorei* plants	Present in *Crinum moorei* bulblets	Reference
1-epideacetylbowdensine	✔	✔	Elgorashi *et al.*, 2001[a]
3-[4'-(8'-aminoethyl)phenoxy]bulbispermine	✔		Elgorashi *et al.*, 2001[a]
acetylcaranine	✔		Viladomat *et al.*, 1997
cherylline	✔	✔	Viladomat *et al.*, 1997 Elgorashi *et al.*, 2001[a]
crinamidine	✔	✔	Lewis, 1996 Elgorashi *et al.*, 2001[a]
crinidine	✔		Watt and Breyer-Brandwijk, 1962
crinine	✔	✔	Lewis, 1996 Elgorashi *et al.*, 2001[a]
Boits crinine	✔		Wildman, 1960
3-*O*-acetylcrinine	✔		Elgorashi *et al.*, 2001[a]
dihydrocrinidine	✔		Watt and Breyer-Brandwijk, 1962
epibuphanisine	✔	✔	Elgorashi *et al.*, 2001[a]
epivittatine	✔		Elgorashi *et al.*, 2001[a]
galanthamine	✔		Viladomat *et al.*, 1997
3-*O*-acetylhamayne	✗	✔	
lycorine	✔	✔	Wildman, 1960 Elgorashi *et al.*, 2001[a]
1-acetyllycorine	✔		Wildman, 1960
1-*O*-acetyllycorine	✔		Viladomat *et al.*, 1997 Elgorashi *et al.*, 2001[a]
9-*O*-methylpseudolycorine	✔		Viladomat *et al.*, 1997
mooreine	✔		Elgorashi *et al.*, 2001[a]
powelline	✔	✔	Lewis, 1996 Elgorashi *et al.*, 2001[a]
tazettine	✔		Viladomat *et al.*, 1997
undulatine	✔	✔	Viladomat *et al.*, 1997 Elgorashi *et al.*, 2001[a]

"One always has to be cautious when comparing alkaloid content of *in vitro* cultures with those of normal plants" (DEMEYER *et al.*, 1992) for the age of the plant and the type of organ analysed may have an influence on the type and quantity of alkaloids produced (DEMEYER *et al.*, 1992; ELGORASHI, 2000). The leaves of *C. moorei,* for example, contained the highest alkaloid levels compared to bulbs, roots and flowering stalks (ELGORASHI, 2000). In *Pancratium biflorum*, Ghosal *et al.* (1984) observed that some Amaryllidaceae alkaloid intermediates were transitory in existence and that certain alkaloids showed rapid gains and losses during the period of intensive growth, especially preceding and during flowering. Seasonal differences in alkaloid content have also been demonstrated for *C. moorei* (ELGORASHI, 2000). Thus, although alkaloids are present in one to two-year-old bulblets, as reported in this study, it is difficult to say with certainty how production compares with field-grown and mature, flowering-size bulbs.

In general, relatively low yields of secondary metabolites are produced in cell and tissue cultures when compared to the parent plant, although they may be produced upon reorganization of the tissues during organogenesis (STABA, 1980). Wild *Narcissus confusus* plants, for example, contain higher concentrations of alkaloids compared to *in vitro* "shoot-clumps" (BERGOÑÓN *et al.*, 1996). And, prancratistatin levels were one tenth of that from field-grown bulbs of *Hymenocallis littoralis* (BACKHAUS *et al.*, 1992). Failure of a cell culture system to produce alkaloids may be a result of any one of the following: (1) all enzymes in the pathway are repressed, (2) some enzymes are repressed, and (3) other factors such as insufficient precursors, lack of storage capacity or the degradation of products (WINK, 1987).

Tissue differentiation and morphogenic effects

Alkaloid biosynthesis is known to be organ- or tissue-specific and thus regulated by development and differentiation (ZHAO *et al.*, 2001). The induction of morphogenesis may improve yields so that "the alkaloid profile may be closer to that *in vivo*" (COLLINGE & YEOMAN, 1986). Undifferentiated calli of *Narcissus confusus*, for example, produced small amounts of galanthamine. However, these levels increased in embryogenic and organogenic cultures (SELLÉS *et al.*, 1999) with embryogenic calli accumulating nearly the same amounts as shoot clumps. The occurrence of Amaryllidaceae alkaloids in newly formed buds of *Narcissus confusus* was at levels comparable to those found in the twin-scales used as explants (SELLÉS *et al.*, 1997[a]). Regenerated bulblets of *Urginea indica* contained both proscillaridin A and scillaren A; bufadienolides characteristic of the parent plants and not found in undifferentiated callus, cell suspension cultures and only in trace amounts in embryogenic and shoot differentiating cultures (JHA *et al.*, 1991).

The alkaloid profile of *N. confusus* cultures also varied with differences in cellular organization (SELLÉS *et al.*, 1999). In meristematic calli haemanthamine (HAEM) was the principal alkaloid, while N-formylnorgalanthamine (N-FNGAL) was the most abundant alkaloid in embryogenic callus. In contrast, galanthamine was the main alkaloid in organogenic tissues.

Explant effects

The source of explant material may affect production. For example, total alkaloid accumulation was higher in the shoot clumps initiated from bulbs rather than seeds of *Narcissus confusus*, such that bulb-derived shoot clumps provide a better source of galanthamine (SELLÉS *et al.*, 1997[b]). For this reason, radioimmunoassay (RIA) techniques have been applied in selecting the most productive cell lines (for alkaloids) of amaryllidaceous species cultured *in vitro* (TANAHASHI *et al.*, 1990; cited by SELLÉS *et al.*, 1997[a]).

Release of alkaloids into the medium

Fewer alkaloids were present in the medium and at concentrations lower than those detected in the whole bulblets of *Crinum moorei*. However, concentrations of the galanthamine-type alkaloids - galanthamine, N-formylnorgalanthamine, haemanthamine and tazettine - were found in even higher concentrations when released into the medium from shoot clumps of *Narcissus confusus* (SELLÉS *et al.*, 1997[b]). Alkaloids are known to be released into the medium, especially in cell culture systems (WINK, 1987). For example, large amounts of berberine are released into the medium of *Thalictrum minus* cell suspension cultures (HARA *et al.*, 1993).

The release of products from plant cells grown in culture is advantageous, since they can be continuously harvested without destroying the cells (PARR *et al.*, 1986). Many substances, including alkaloids of pharmaceutical importance, are naturally released by cells *in vitro*. This may occur via cell lysis or by light sensitive transport mechanisms (KIM *et al.*, 1988). The transport of alkaloids into the medium may occur via an active or passive mechanism driven by light-sensitive pH differences between the vacuole and the medium (KIM *et al.*, 1988). It is thought that secondary products are released through an endogenous secretory mechanism. Alternatively, they are stored intracellularly in vacuoles or vesicles in the cytoplasm (BURBIDGE, 1993), as in *Cinchona ledgeriana* (PARR *et al.*, 1986). Differences in the mechanism of alkaloid transport may be attributed to the nature of the molecule itself. Uncharged alkaloid molecules are able to freely diffuse across membranes, while protonated alkaloid cations cannot. Their movement is, perhaps, pH dependent. "Ion traps" result in the accumulation of alkaloids in acidic compartments or else they may bind to intracellular

materials such as phenolics (RENAUDIN, 1981; cited by PARR *et al.*, 1986). To induce the release of stored alkaloids, both the plasmalemma and tonoplast need to be permeabilized (PARR *et al.*, 1986).

THE EFFECTS OF PHYSICAL AND CHEMICAL FACTORS ON ALKALOID PRODUCTION

Introduction

Secondary metabolites (including alkaloids) are thought to function as defence substances (WINK, 1987). In an environment that provides artificial conditions for plant growth, "it is possible that the imposition of such conditions may induce stresses which contribute to changes in the levels of secondary metabolite formation" (RUDGE & MORRIS, 1986). Thus secondary metabolite production, *in vitro*, can similarly be triggered by environmental stresses; providing useful clues to the sensitivity of secondary metabolites to environmental changes (RUDGE & MORRIS, 1986).

However, biosynthesis using plant tissue cultures is just as dependent on the close relationship between production and differentiation as it is to environmental influences like light and media constituents, particularly plant hormones (BUTCHER, 1977). For in the intact plant, the degree of tissue differentiation is one of the most important factors that influences compound production. Secondary product biosynthesis may even be part of the differentiation process (BUTCHER, 1977).

The modification of secondary metabolite production *in vitro* is primarily concerned with increased production, particularly of those compounds of economic importance (VERPOORTE, 1998). Strategies that can be used to improve production *in vitro* include: (1) selection and screening of high yielding lines; (2) optimizing conditions for growth, secondary metabolite synthesis and product release e.g. appropriate medium design and lighting; (3) differentiation; (4) immobilization of cells; (5) elicitation and (6) genetic manipulation through biotransformation (ANDERSON *et al.*, 1986; DICOSMO & MISAWA, 1995; GIULIETTI & ERTOLA, 1999). It is important to bear in mind that not all steps in the biosynthetic pathway are regulated solely by the presence or activity of enzymes, but also by their compartmentation and transport of intermediates (VERPOORTE, 1998).

Explant source

Explants derived from different parts of alkaloid-synthesizing plants are capable of producing alkaloids in culture (MANTELL & SMITH, 1983). These are identical to those produced in the

whole plant. It is generally assumed that cultures established from high-yielding plants will produce high product yields. This has been shown for alkaloid production in *Catharanthus roseus* (FOWLER, 1983). In other instances, there is no correlation as some cultures are reported to produce even higher alkaloid levels than in the intact plant (CORDUAN, 1975; cited by MANTELL & SMITH, 1983). Lack of tissue differentiation in callus and liquid cultures may be responsible for differences in alkaloid production between the parent plant and cell culture system. In those cases where secondary metabolite production exceeds that of the parent plant, synthesis takes place in root tissue (WINK, 1987); suggesting that some degree of tissue differentiation is a necessary prerequisite for metabolite production, as in the case of morphine (TABATA, 1977) and the alkaloid vindoline (ZHAO *et al.*, 2001). Interestingly, organ differentiation from callus nearly always triggers renewed synthesis e.g. alkaloids of morphine (ROBERTS, 1988) and hyoscymine. Alkaloid levels were low in callus cultures of *Coptis japonica*. However, when plantlets were regenerated from the callus, alkaloid levels returned to normal (NICKELL, 1980). Thus with differentiation, there is controlled gene expression (WINK, 1987).

Light

Light affects, not only growth and organogenesis, but also the formation of plant products including both primary and secondary metabolites (KIM *et al.*, 1988). Light stimulates the formation of a wide range of secondary products in cell cultures e.g. carotenoids, flavonoids, anthocyanins, polyphenols and plastoquinones (TABATA, 1977; SEIBERT & KADKADE, 1980; cited by HOBBS & YEOMAN, 1991; KURATA *et al.*, 1994). The type of light may also influence secondary metabolite synthesis in culture. For example, "Cool White" light stimulated alkaloid biosynthesis in *C. roseus* cell cultures (MANTELL & SMITH, 1983). Where alkaloid production depends on the availability of light e.g. for lupine alkaloids (WINK, 1987), it may be that there is a better supply of lysine; that enzymes required for biosynthesis function at an optimum pH of 8 (as in the chloroplast stroma) or that enzymes are activated by reduced thioredoxin which is produced only in the light (WINK, 1987). In contrast, the inhibitory effects of light have been documented for *Scopolia parviflora* cultures as suppressing alkaloid formation (MANTELL & SMITH, 1983), for a higher content of hyoscyamine and scopolamine alkaloids was found in dark-grown callus (DEMEYER *et al.*, 1992). This response may be attributed to the repression of photodegradation of certain metabolites and / or enzymes (MANTELL & SMITH, 1983).

Media constituents

One promising strategy for improving yields is to manipulate the medium (GIULIETTI & ERTOLA, 1999). This may be done in two phases; by firstly using a growth medium to increase biomass and, secondly, a production medium to enhance secondary metabolite synthesis (GIULIETTI & ERTOLA, 1999). Media constituents known to affect secondary metabolite synthesis include: carbon, nitrogen, the carbon-nitrogen ratio, inorganic phosphate and elicitors (GIULIETTI & ERTOLA, 1999) as well as a wide range of plant growth regulators.

Plant growth regulators

Growth regulators have a remarkable effect, not only on growth and differentiation, but also on secondary metabolites; effectively triggering secondary metabolism *in vivo* (MANTELL & SMITH, 1983; EL-BAHR *et al.*, 1989). However, this may depend on both the type and concentration of growth regulators used (TABATA, 1977; EL-BAHR *et al.*, 1989).

Nutrients

Nitrogen is supplied by NO_3^- and NH_4^+. When supplied separately, growth and the synthesis of secondary metabolites are affected negatively (GIULIETTI & ERTOLA, 1999). Phosphate (inorganic) limitation has, in several instances, been used to elicit an increase in secondary product accumulation. For example, a low phosphate concentration stimulated the production of alkaloids in *Catharanthus roseus* (GIULIETTI & ERTOLA, 1999). Generally, those factors which support active growth in culture limit secondary metabolite production (MANTELL & SMITH, 1983).

Carbon source

Sugar levels can influence metabolism, although "not always in a predictable way" (FOWLER, 1983; GIULIETTI & ERTOLA, 1999). In general, an increase in the level of sucrose increases yields of secondary metabolites (MANTELL & SMITH, 1983). For example, an increase in sucrose to levels above 3% led to substantial increases in alkaloid yields of *C. roseus* (ZENK *et al.*, 1977; cited by FOWLER, 1983). Different levels of sucrose also affected the alkaloid profile of shoot clumps of *Narcissus confusus* and the secretion of alkaloids into the medium (SELLÉS *et al.*, 1997[b]). High (9%) levels of sucrose benefited the production of galanthamine.

Precursors and elicitors

Elicitors are abiotic compounds (e.g. heavy metal salts or agents like UV radiation) or extracts derived from organisms that trigger the synthesis of secondary metabolites (BURBIDGE, 1994; GIULIETTI & ERTOLA, 1999). Cell cultures treated with fungi and bacteria sometimes show an increased production of alkaloids (WINK, 1987). Shoot-clump cultures of *Narcissus confusus* failed to show an increase in galanthamine production as a consequence of adding *trans*-cinnamic acid - a precursor - to the medium (BERGOÑÓN *et al.*, 1996). Although production was inhibited, another alkaloid in the same biosynthetic pathway, *N*-formyl-norgalathamine, showed increased levels (BERGOÑÓN *et al.*, 1996). Another advantage of using elicitors is that products are often released into the medium (GIULIETTI & ERTOLA, 1999).

Thus, increasing secondary metabolite synthesis can be achieved by optimizing growth and the culture medium as well as through the use of elicitors and metabolic engineering (GIULIETTI & ERTOLA, 1999).

Various factors, relating to the culture environment, can further be manipulated to facilitate the release of secondary compounds (PARR *et al.*, 1986).

Aims

In vivo studies have shown that the content of galanthamine in *Leucojum aestivum*, for example, is eco-geographically determined and, therefore, can be controlled (GORINOVA *et al.*, 1993). The aim of this study was to determine whether physical factors, such as light, and media components, like charcoal and cytokinins, could be used to improve alkaloid production in an *in vitro* system. Light and cytokinin effects on alkaloid production are well-documented. There are, however, no reports for the effects of activated charcoal on secondary metabolite synthesis. Since charcoal is frequently added to tissue culture media for various reasons (PAN & VAN STADEN, 1998), and particularly for inducing bulblet formation and superior growth (this study), its effect on alkaloid synthesis and release into the medium was examined.

Materials and methods

Plant material

Bulblets subjected to the following physical and nutritional conditions were used for alkaloid analysis: (1) dark; (2) BA-supplemented media (2 mg ℓ^{-1}); (3) charcoal-supplemented media

(5 g ℓ^{-1}) and for comparative purposes, the control bulblets were kept in the light on MS media with no hormone or charcoal supplements. The bulblets and modified MS media were analysed separately.

Extraction, identification and quantification

The same preparative and analytical techniques were used as reported earlier in this chapter (see "Extraction, identification and quantification of *Crinum* alkaloids"). The alkaloid concentration of each treatment represents the mean for three replicates. As a precautionary measure, each sample was injected twice.

Results

A comparison of media and other physical effects on alkaloid production showed that light was essential, for almost no alkaloids were synthesized in the dark (Figure 8.2). Crinamidine, powelline and undulatine were present, although at much lower concentrations than in the light (control). Higher concentrations of powelline, 1-epideacetylbowdensine and 3-*O*-acetylhamayne were recorded in BA-supplemented media compared to the control (Figure 8.2). Greater amounts of cherylline, crinine, epibuphanisine, lycorine and undulatine were present in media containing activated charcoal (Figure 8.2). Only cherylline occurred in significantly greater amounts compared to the control (Figure 8.2). Thus, most alkaloids were produced at their highest levels in media containing activated charcoal, followed by BA-supplemented media and then charcoal-free media. The inclusion of BA (at 2 mg ℓ^{-1}) or charcoal (5 g ℓ^{-1}), thus, has a stimulatory effect by increasing the production of *Crinum* alkaloids in bulblets grown *in vitro*.

Crinine and powelline were released into the medium containing BA, although this was not statistically significantly different to the control (Figure 8.3). Smaller amounts were present compared to the quantities of these alkaloids extracted from the bulblets. Undulatine was detected in the control medium (i.e. without BA or charcoal) but at levels not significantly different to the control. No other alkaloids were detected in the medium of bulblets grown in the dark or on charcoal-containing medium (Figure 8.3). The effect of BA, in the medium, was to stimulate the release of specific alkaloids from the bulblets of *C. moorei*.

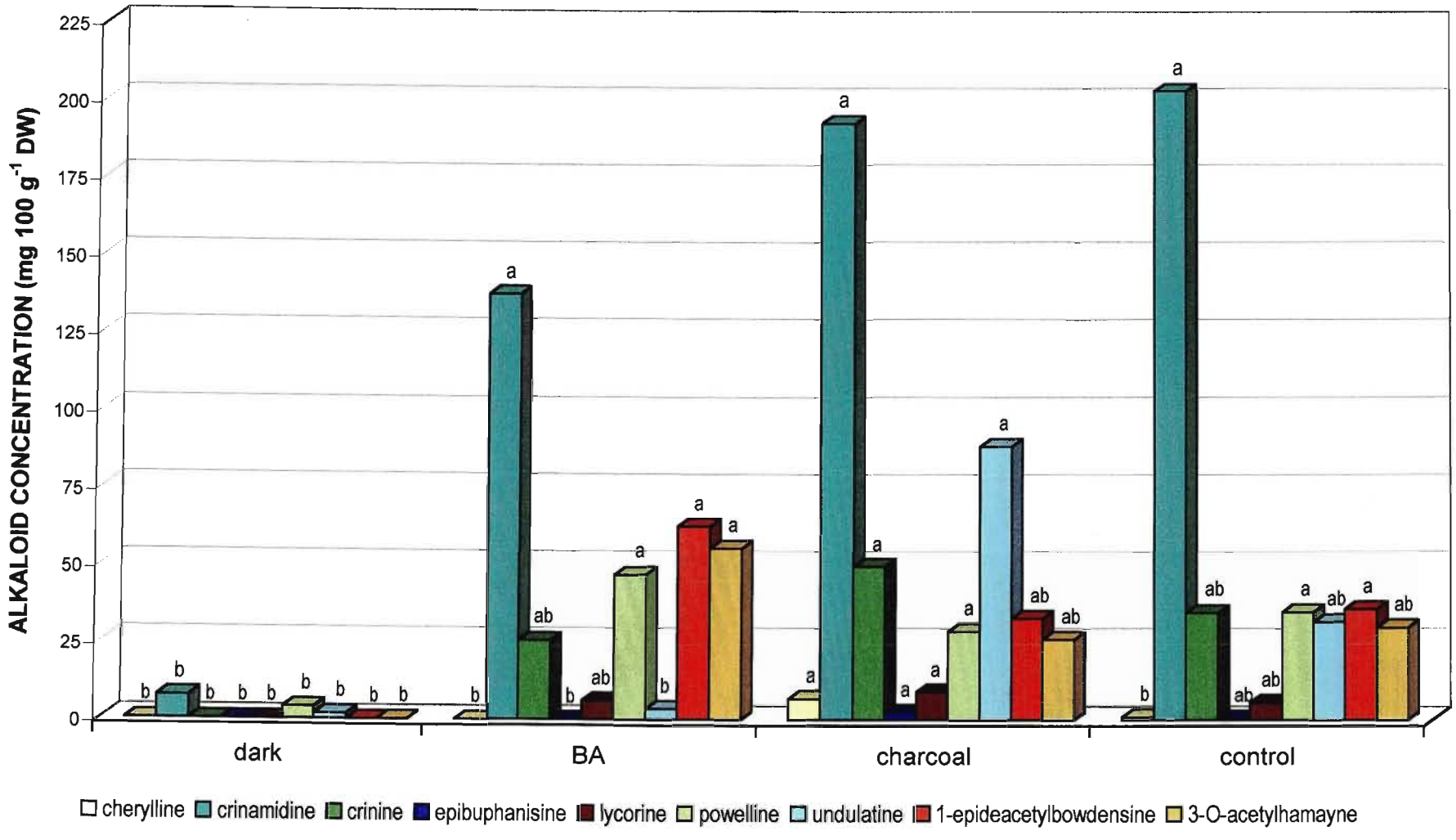


Figure 8.2: Effect of dark, BA and charcoal on alkaloid levels in *C. moorei* bulblets
Different letters show significant differences between treatments at the 5% level (ANOVA)

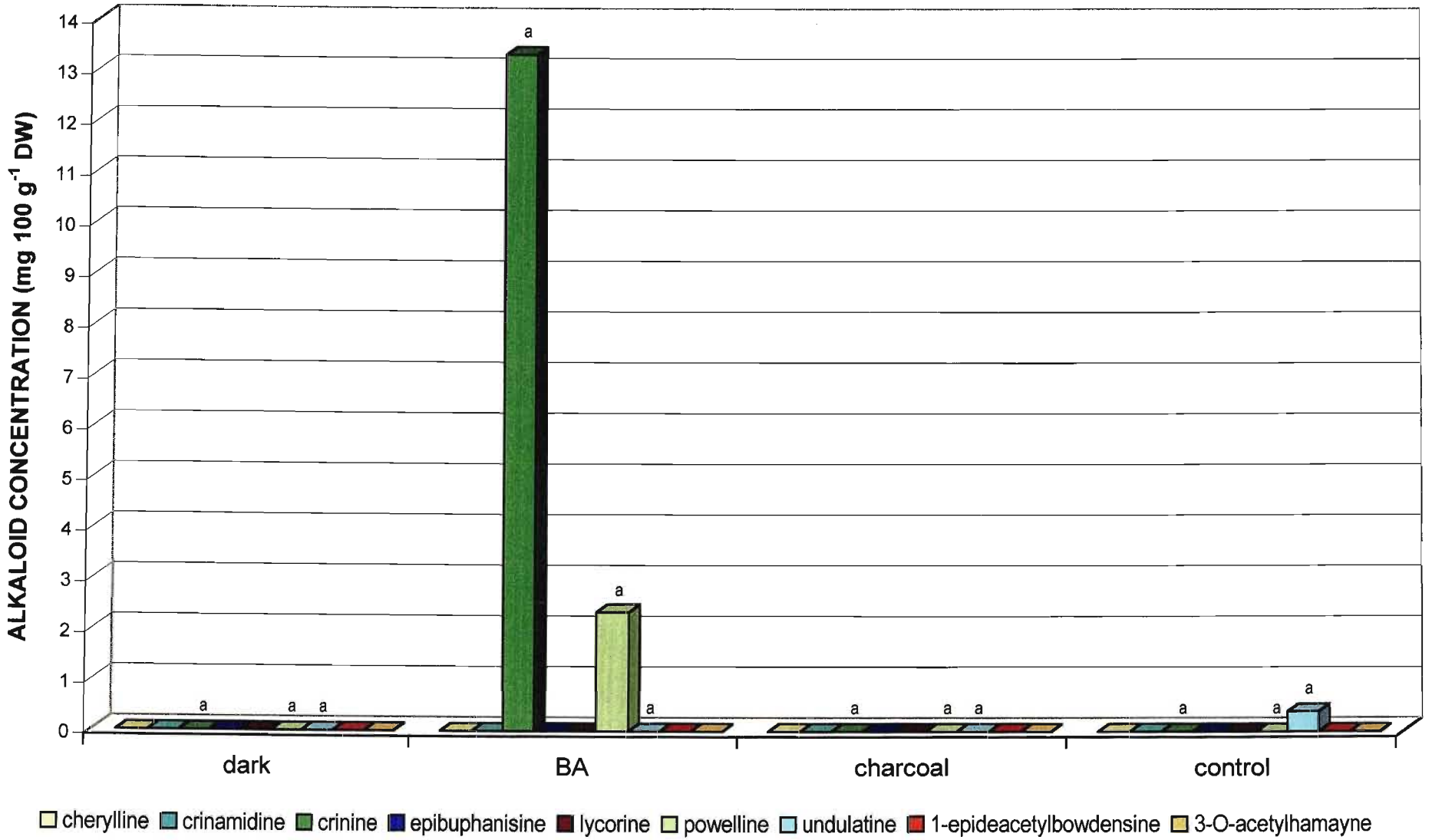


Figure 8.3: Effect of dark, BA and charcoal on alkaloid release into the MS medium supporting the growth of *C. moorei* bulblets
Different letters show significant differences between treatments at the 5% level (ANOVA)

Discussion

The effect of light on alkaloid production

In general, no *Crinum* alkaloids were produced in tissues stored in the dark. The absence of alkaloids from cell cultures grown in the dark has also been reported for *Lupinus polyphyllus* and *Cytisus scoparius* (WINK, 1987).

Light, therefore, is an important trigger for secondary metabolite biosynthesis in several plant species where synthesis does not take place in the dark (KIM *et al.*, 1988). The presence of light, for example, enhanced berberine synthesis in suspension cultures of *Coscinium fenestratum* (NAIR *et al.*, 1992), even when supplied continuously, and in *Thalictrum rugosum* (NAIR *et al.*, 1992). The purine alkaloids, caffeine and theobromine, were produced in *Coffea arabica* cell cultures grown in the light (KURATA *et al.*, 1994). Vindoline and serpentine biosynthesis in *Catharanthus roseus* callus cultures were also promoted by light (ZHAO *et al.*, 2001). Vindoline content was, in fact, 3 - 4 times higher in the illuminated cultures.

Galanthamine production, however, was similar both in the light and dark, although that of NFGAL, occurred preferentially in the dark (BERGOÑÓN *et al.*, 1996).

The absence of alkaloids in dark-grown bulblets may be related to the absence of chlorophyll. There appears to be a correlation between alkaloid production and chlorophyll content, as demonstrated in cell cultures of *L. polyphyllus* and *Cytisus scoparius* (WINK, 1987), for only green photomixotrophic cultures were capable of producing lupanine or sparteine alkaloids. This suggests that the ability to synthesize alkaloids, in this instance, is a function of the chloroplast (WINK, 1987). Zhao *et al.* (2001) maintain that it is in developed chloroplasts that many of the important precursors for the synthesis of indole alkaloids are manufactured. The role of light is to induce differentiation, including the activation of genes and morphogenesis e.g. phloem and xylem. Only when cell cultures resemble leaf mesophyll cells, do tissue-specific promoters activate biosynthesis. Increased alkaloid production in the light may result from: (1) an improved supply of lysine in the light; (2) the creation of a pH of 8 in the chloroplast stroma which is necessary for the proper functioning of enzymes involved in alkaloid biosynthesis, and (3) the regeneration of thioredoxin which activates the enzymes (WINK, 1987). The stimulating effect of light is probably a result of chloroplasts supplying biosynthetic intermediates (DALTON & PEEL, 1983; cited by HOBBS & YEOMAN, 1991). In the case of *Catharanthus roseus*, light was thought to regulate alkaloid biosynthesis and accumulation by influencing peroxidase activity and chloroplast development in callus cultures (ZHAO *et al.*, 2001). Basic peroxidases are involved in the metabolism of indole alkaloids

while acidic forms are necessary for differentiation and development (ZHAO *et al.*, 2001). Light stimulates their activity (ZHAO *et al.*, 2001).

Thus, for *Crinum moorei*, there is an alteration in the pattern of product synthesis for light- and dark-grown cultures.

The effect of light on the release of alkaloids into the medium

In addition to the inductive effects of light on secondary metabolite production, light may also regulate the mechanisms for secretion (KIM *et al.*, 1988). For *Crinum moorei*, dark conditions suppressed the release of alkaloids into the medium (compared to the control). Light conditions also affected the release of alkaloids into the medium in the case of *N. confusus*. Greater amounts of galanthamine were released in the light as opposed to NFGAL, HAEM and tazettine (TZ) which appeared in higher quantities in the dark (BERGOÑÓN *et al.*, 1996). In contrast, cell cultures of *Catharanthus roseus* produced ajmalicin and serpentine as extracellular products in the dark but at decreased levels under light conditions (DRAPEAU *et al.*, 1987; cited by KIM *et al.*, 1988). The secretion of berberine from cell cultures of *Thalictrum rugosum* was also significantly suppressed by light (KIM *et al.*, 1988). Drapeau *et al.* (1988) postulate that pH differences caused by sensitivity to light may account for the transport of alkaloids into the medium of *C. roseus* cultures.

The effect of cytokinins on alkaloid production

The role of cytokinins in regulating secondary metabolism is less clear; they either have no influence or stimulate or inhibit the process (EL-BAHR *et al.*, 1989; DECENDIT *et al.*, 1992).

Cytokinins stimulate or enhance the production of a wide range of secondary metabolites such as nicotine (*Nicotinana tabacum)*; tobacco coumarins (DECENDIT *et al.*, 1992); phenolic compounds like anthocyanins and betacyanins (DECENDIT *et al.*, 1992); the carotenoid rhodoxanthin in *Ricinus* cell cultures (DECENDIT *et al.*, 1992); catechins; proanthocyanidins and lignins (HARA *et al.*, 1993) as well as alkaloids (ROBERTS, 1988).

In studying the effects of cytokinins on alkaloid biosynthesis, cytokinins (and ethylene) were found to up-regulate alkaloid production in suspension cultures of *Catharanthus roseus*, that is, when they were supplied exogenously (YAHIA *et al.*, 1998). Their stimulatory effect occurs only upon removal of auxin from the medium (DECENDIT *et al.*, 1992). Furoquinoline alkaloid accumulation in cell cultures of *Fagara zanthoxyloides* is also dependent on the presence of exogenously supplied cytokinins in the form of BA. In its absence, alkaloid production was

nine times lower than that observed in control cultures; thus confirming its stimulatory effect (COUILLEROT *et al.*, 1996). Kinetin, rather than BA, was found to promote alkaloid production in *Scopolia maxima* cultures (MANTELL & SMITH, 1983).

The use of cytokinins in media to promote growth has the added benefit of maintaining alkaloid productivity in tissue cultures capable of producing isoquinoline-like alkaloids (HARA *et al.*,1993). For example, BA and kinetin were found to benefit morphinan alkaloid production (ROBERTS, 1988) i.e. codeine production was enhanced, while high levels of kinetin significantly improved production of quantities of codeine and thebaine in green callus (ROBERTS, 1988). For berberine production, the addition of BA resulted in the rapid conversion of the precursor L-tyrosine into berberine. This effect appears to be unique in that it is one of the few instances where BA is known to induce alkaloid biosynthesis (HARA *et al.*, 1993). Including BA in the medium in this study resulted in a similar effect, that is, it increased production of powelline, 3-*O*-acetylhamayne and 1-epideacetylbowdensine.

Some cytokinins appear to be more active than others. For example, BA, and trans-zeatin, were shown to increase indole alkaloid accumulation in *C. roseus* cultures more so than kinetin (DECENDIT *et al.*, 1992). In contrast, kinetin alone gave the highest amount of hyoscine and hyoascyamine content for *Datura stramonium* cultured *in vitro* (EL-BAHR *et al.*, 1989); that is, in both leaf and stem callus where total alkaloid levels were increased by 100 - 117%.

In other reports, cytokinins were shown to retard the production of secondary metabolites. Improved hyoscyamine and scopolamine alkaloid production, for example, were only achieved in media containing a low content of BA and 2,4-D (DEMEYER *et al.*, 1992). At high concentrations, kinetin inhibited alkaloid production in callus cultures of *Datura tatula* (MANTELL & SMITH, 1983) and the production of indole alkaloids by *Cinchona legeriana* (HARKES *et al.*, 1985; cited by EL-BAHR *et al.*, 1989). In the callus of *D. innoxia*, the effect of cytokinins was also to decrease alkaloid levels (SZOKE *et al.*, 1982; cited by DEMEYER *et al.*, 1992). There are other instances where higher levels of alkaloids are produced in culture media free of plant growth regulators or in habituated cell lines e.g. *Catharathus roseus* and *Papaver somniferum* (COUILLEROT *et al.*, 1996).

On the contrary, cytokinins had no effect on alkaloid concentrations in cultures of *Cinchona ledgeriana* (HARKES *et al.*, 1985; cited by DEMEYER *et al.*, 1992).

Cytokinins are known to be important in the repression or derepression of enzyme production and in influencing the rate of product turnover (STABA, 1980). Their inductive effects on alkaloid biosynthesis, therefore, may be the indirect result of key enzyme activation, vesicle

formation or ion influx. Hara *et al.* (1993) suggest that the induction of berberine synthesis by BA is via hormonal activation of enzymatic reactions subsequent to the formation of amines in the biosynthesis of berberine. For although the precursors are present even in media lacking BA, their conversion into berberine required that cytokinins, like BA, were present (HARA *et al.*, 1993). It is also possible that cytokinins are involved in the formation of subcellular vesicles, since it is here that the final steps in the biosynthetic pathway take place and alkaloids are accumulated (HARA *et al.*, 1993). In the case of alkaloid accumulation in *Catharanthus roseus* cell cultures, there is strong evidence that the enhancing effect of BA is mediated through an influx of Ca^{2+} ions (DECENDIT *et al.*, 1992).

The effect of BA on the release of alkaloids into the medium

In this study, BA stimulated the release of specific alkaloids from the bulblets of *C. moorei*. Such responses are poorly recorded in the literature. Where it does occur is mostly in suspension cultures or liquid media. The development of a deep yellow colour in liquid media containing cultures of *Coscinium fenetratum*, for example, was due to the release of the alkaloid berberine (NAIR *et al.*, 1992). Although hormonal conditions were suggested to play a crucial role in berberine production, their use and effect on alkaloid release into the medium was not addressed.

The effect of activated charcoal on alkaloid production

High levels of cherylline, crinine, epibuphanisine, lycorine and undulatine were found in bulblets grown on media containing activated charcoal.

By reducing the light at the base of shoots, charcoal provides an environment conducive to the accumulation of photosensitive auxins and their co-factors (PAN & VAN STADEN, 1998) and, in this manner, promotes root development (PAN & VAN STADEN, 1998). Since alkaloids of the Amaryllidaceae occur in roots, sometimes even at their highest levels (SELLÉS *et al.*, 1997[a]), it thus follows that alkaloid production and accumulation may be enhanced by darkening the medium.

It is thought that adsorbed minerals are gradually released from charcoal and as such are made available to plant tissues by active uptake (PAN & VAN STADEN, 1998). Elements known to be present in activated charcoal include: Cu, Fe, K, Mg, Mo and Zn (ERNST, 1975; JOHANSSON *et al.*, 1990; cited by PAN & VAN STADEN, 1998), while other inorganic ions and metals, like NH_4, NO_2 and NO_3 have also been recorded in water treated with activated charcoal (JOHANSSON *et al.*, 1990; cited by PAN & VAN STADEN, 1998). NH_4, NO_2 and

NO_3 as well as Cu, Fe, K, Mg, Mo and Zn are released from activated charcoal in significant amounts (JOHANSSON *et al.*, 1990; cited by PAN & VAN STADEN, 1998). It is therefore likely that charcoal acts like colloidal soil particles to which many materials are absorbed or desorbed (PAN & VAN STADEN, 1998). Gorinova *et al.* (1993) showed that galanthamine biosynthesis *in vivo* could be controlled by the level of soil fertility, since a relationship existed between the galanthamine content in the plant and the chemical composition of the soil. High potassium (K) and boron (B) concentrations in the soil corresponded to the highest recorded levels of galanthamine in the leaves and bulbs. Moreover, the ratio of K to B was found to be favourable for the utilization and transport of assimilates in the plant and a guarantee against stress (mainly low soil moisture) that may have occurred in the environment (GORINOVA *et al.*, 1993). Good reserves of N, Mg, Mo, Zn, Fe and Cu in the soil were also associated with high concentrations of galanthamine (GORINOVA *et al.*, 1993).

Glucosyloxy alkaloids from the amaryllidaceous plant *Pancratium biflorum* (GHOSAL *et al.*, 1984), and their aglucones, were reported to form stable complexes with divalent metal ions like Cu^{2+}, Zn^{2+} and Fe^{2+}. These were then translocated from the rhizosphere to the aerial parts of the plant (GHOSAL *et al.*, 1984). Interestingly, lycorine-1-*O*-β-D-glucoside complexed with Cu^{2+} was found to produce significant root growth in *P. biflorum* although the complex involving lycorine inhibited the growth of roots (GHOSAL *et al.*, 1984). "Alkaloids are known to elicit their activities by modulating the transport of metal ions across biological membranes" (GHOSAL *et al.*, 1984).

It is probable that the presence of activated charcoal in the medium makes available substances, much like soil does, and that these elements influence the synthesis of alkaloids by taking part in enzyme reactions (GORINOVA *et al.*, 1993). Alternatively, they may facilitate the formation of stable alkaloid-ion complexes which are then more easily detected in above ground plant parts.

CONCLUSIONS

Nine alkaloids, of the Amaryllidaceae type, were identified in the bulblets of *Crinum moorei*; three of which were released into the medium supporting the growth of the bulblets. A comparison of media and other physical effects on alkaloid synthesis *in vitro*, showed that light was essential and that the inclusion of the cytokinin BA or activated charcoal stimulated the production of specific alkaloids.

This is the first report of *in vitro* alkaloid synthesis for *Crinum* species. Not only does this hold prospects for extraction from plant sources other than those in the wild, but also as a system

for investigating and manipulating factors within the culture environment for improved product synthesis and recovery.

Phytochemical studies of this nature are of increasing importance because much of the planet's diversity is disappearing as a result of human movement and increase in the world's population (RAFFAUF, 1996).

Conclusions

As an alternative to conventional propagation by seed and offsets, micropropagation of *Crinum moorei* has many advantages. The bulb, with all its meristematic tissue situated at the base of scales, can be induced to produce shoots upon cutting. Tissue culture exploits this natural mechanism of axillary growth by using twin-scales. Each consists of two adjacent scales joined at the base by a small segment of the basal plate. It is in the axes of the scales that shoots develop. Thus for every explant, and there may potentially be as many as two hundred in a mature bulb of 97 mm, a bulblet will develop as the shoot becomes swollen at its base. This is the final product in micropropagation schemes involving bulbous plants. Bulblet production can be optimized *in vitro* by altering the media constituents and the culture environment. In this study, several growth-promoting factors were identified, namely: elevated sucrose levels (6%), activated charcoal, light and a temperature of 25°C. Tissue-dependent factors i.e. explants larger than 3 mm at their base, and from an intermediate position in the parent bulb, also affected bulblet regeneration and growth from the twin-scales. There appeared to be no seasonal effects, as explants could be excised and produced plantlets at any time of the year. Under optimal conditions each twin-scale formed a bulblet within 17 weeks. Those with a diameter of 5 mm or more, could be split vertically in half to produce secondary bulblets on a multiplication medium. In a liquid-shake culture system growth rates improved. Meristematic clusters formed in response to continuous agitation and supplementation with 10 mg ℓ^{-1} BA. These could be separated into approximately 0.100 g propagules for further bulblet development on solid MS media containing 5 g ℓ^{-1} activated charcoal or 6% sucrose. Compared to multiplication on gelled media, where multiplication of *in vitro* grown material was slow, especially with each successive subculture passage, multiplication of the propagules in liquid-shake culture was approximately twice as productive and shortened the time necessary to produce fully differentiated bulblets.

In addition to twin-scaling, floral stems provide an alternative source of explants that may ultimately form shoots. However, explantation is restricted to the flowering season and shoot production is limited by a complex of factors relating to the age of the floral stem, position effects and hormones.

Although somatic embryogenesis is another likely means of mass propagating bulbs, the technique, as developed in this study, offers few advantages over multiplication in a liquid-shake culture system since embryo induction and plantlet regeneration occurred infrequently.

Micropropagated bulblets were found to contain Amaryllidaceae alkaloids of the *Crinum* type. By manipulating the cultural environment, either through media or light effects, it was possible to further improve product yields. This may have important implications for the production of biologically active alkaloids, such as galanthamine.

Micropropagation of *Crinum moorei*, which was successfully achieved, thus offers many advantages to conventional propagation. Production occurs year-round and multiplication rates are high using a liquid-shake culture system. In future, this will be the most efficient means of scaling-up the process to automated bioreactor culture, not only for bulblet production but for the synthesis of secondary metabolites of pharmaceutical importance as well.

References

ABD-EL-HAFIZ, M.A. 1990. Two keto alcohols from *Crinum augustum*. *Phytochemistry* 29: 3936-3938.

ABD-EL-HAFIZ, M.A. 1991. Aliphatic hydroxyketones from *Crinum augustum*. *Phytochemistry* 30(9): 3127-3129.

ABD-EL-HAFIZ, M.A., RAMADAN, M.A., JUNG, M.L., BECK, J.P. and ANTON, R. 1991. Cytotoxic activity of Amaryllidaceae alkaloids from *Crinum augustum* and *Crinum bulbispermum*. *Planta Medica* 57(2): 437-439.

ABBOTT, A.J. and ATKIN, R.K. 1987. Improving vegetatively propagated crops. Academic Press, London. ISBN 0-12-041410-4.

ABBOTT, A.J. and BELCHER, A.R. 1986. Potato tuber formation *in vitro*. In: Withers, L.A. and ALDERSON, P.G. (Eds). Plant tissue culture and its agricultural applications. Butterworth, London, pp. 113-122. ISBN 0-407-00921-3.

ADELBERG, J. 1998. Regeneration and frequency of tetraploid variants of *Cucumis metuliferus* are affected by explant induction on semi-solid medium versus the liquid/membrane system. *Plant Cell Reports* 17(3): 225-229.

ADESANYA, S.A., OLUGBADE, T.A., ODEBIYI, O.O. and ALADESANMI, J.A. 1992. Antibacterial alkaloids in *Crinum jagus*. *International Journal of Pharmacognosy* 30(4): 303-307.

AHMAD, M. 1996. Cytotoxic activity of the leaf extract of *Crinum asiaticum* Linn. *Australian Journal of Medical Herbalism* 8(1): 3-6.

AITKEN-CHRISTIE, J. 1991. Automation. In: Debergh, P.C. and Zimmerman, R.H. (Eds). Micropropagation: Technology and Application, pp. 363-388. Kluwer Academic Publishers. ISBN 0-7923-0818-2.

AKITA, M. and TAKAYAMA, S. 1988. Mass propagation of potato tubers using jar fermentor techniques. *Acta Horticulturae* 230: 55-61.

ALBRECHT, C.F., THERON, E.J. and BURGER, I. 1996. Detection of synergism between extracts of a *Crinum* sp. and *Becium* sp. - cytotoxicity of cancer cells *in vitro*. *South African Association of Botanists Annual Congress, January 1996.*

ALDERSON, P.G. and RICE, R.D. 1986. Propagation of bulbs from floral stem tissues. In: Withers, L.A. and Alderson, P.G. (Eds). 1986. Plant tissue culture and its agricultural applications. Butterworth, London, pp. 91-97. ISBN 0-407-00921-3.

ALEXANDER, M. 1994. *Crinum moorei:* These plants make a spectacular show in the summer garden. *South African Garden and Home* (May, 1994) 34005: 148.

ALFERMANN, A.W. and PETERSEN, M. 1995. Natural product formation by plant cell biotechnology. *Plant Cell, Tissue and Organ Culture* 43: 199-205.

ALI, A.A., EL-MOGHAZY, A.M., ROSS, S.A. and EL-SHANAWANY, M.A. 1981. Phytochemical studies on some Amaryllidaceae cultivated in Egypt. *Fitoterpia* 52(5): 209-212.

ALI, A.A., SAYED, H.M., ABDALLA, O.M. and STEGLICH, W. 1988. Flavans from *Crinum americanum*. *Pharmazie* 43(4): 295-296.

ALVARD, D., COTE, F. and TEISSON, C. 1993. Comparison of methods of liquid medium culture for banana micropropagation. *Plant Cell, Tissue and Organ Culture* 32: 55-60.

AMMIRATO, P.V. 1983. Embryogenesis. In: Evans, D.A., Sharp, W.R., Ammirato, P.V. and Yamada, Y. (Eds). Handbook of plant cell culture. Volume 1. Techniques for propagation and breeding. Macmillan Publishing Co., New York, pp. 82-123. ISBN 0-02-949230-0.

AMMIRATO, P.V. 1987. Organizational events during somatic embryogenesis. In: Green, C.E., Somers, D.A., Hackett, W.P. and Biesboer, D.D. (Eds). Plant tissue and cell culture. Alan R. Liss, Inc., New York, pp. 57-81. ISBN 0-8451-1802-1.

ANDERSON, L.A., PHILLIPSON, J.D. and ROBERTS, M.F. 1986. Aspects of alkaloid production by plant cell cultures. In: Morris, P., Scragg, A.H., Stafford, A. and Fowler, M.W. (Eds). Secondary metabolism in plant cell cultures. Cambridge University Press, Cambridge. ISBN 0-521-32889-6.

ANTOUN, M.D., MENDOZA, N.T., RIOS, Y.R., PROCTOR, G.R., WICKRAMARATNE, D.B.N., PEZZUTO, J.M. and KINGHORN, A.D. 1993. Cytotoxicity of *Hymenocallis expansa* alkaloids. *Journal of Natural Products. Lloydia* 56(8): 1423-1425.

ARCHER, R.H. 1997. *Crinum acaule, C. minimum* and *C. parvibulbosum* in southern Africa. *Herbertia* 52: 90-94.

ARZATEFERNANDEZ, A.M., NAKAZAKI, T., OKUMOTO, Y. and TANISAKA, T. 1997. Efficient callus induction and plant regeneration from filaments with anther in Lily (*Lilium longiflorum* Thunb.). *Plant Cell Reports* 16(12): 836-840.

AULT, J.R. 1995. *In vitro* propagation of *Eucomis autumnalis, E. comosa* and *E. zambesiaca* by twin-scaling. *HortScience* 30(7): 1441-1442.

BACH, A. 1992. Somatic embryogenesis from zygotic embryos and meristems of *Freesia hybrida*. *Acta Horticulturae* 325: 429-434.

BACKHAUS, R.A., PETTIT, G.R., HUANG, D.S., PETTIT, G.R., GROSZEK, G., ODGERS, J.C., HO, J. and MEEROW, A. 1992. Biosynthesis of the antineoplastic pancratistatin following tissue culture of *Hymenocallis littoralis* (Amaryllidaceae). *Acta Horticulturae* 306: 364-366.

BAJAJ, Y.P.S., FURMANOWA, M. and OLSZOWSKA, O. 1988. Biotechnology of the micropropagation of medicinal and aromatic plants. In: Bajaj, Y.P.S. (Ed). Biotechnology in agriculture and forestry. Volume 4. Medicinal and aromatic plants. I. Springer-Verlag, Berlin, pp. 60-103. ISBN 3-540-18414-7.

BALZARINI, J., SCHOLS, D., NEYTS, J., VAN DAMME, E., PEUMAN, S.W. and DE CLERCQ, E. 1991. Alpha-(1-3) and alpha-(1-6)-D-mannose-specific plant lectins are markedly inhibitory to human immunodeficiency virus and cytomegalovirus infections *in vitro*. *Antimicrobial Agents and Chemotherapy* 35(3): 410-416.

BANG, M. 1996. Medicinal gardens become vibrant community focus. *The Natal Witness*, August.

BASTIDA, J., CODINA, C., PEETERS, P., RUBIRALTA, M., OROZCO, M., LUQUE, F.J. and CHHABRA, S.C. 1995. Alkaloids from *Crinum kirkii*. *Phytochemistry* 40(4): 1291-1293.

BAYER, A. 1979. Flower paintings of Katharine Saunders. Botanical and biographical notes and explanations. Tongaat Group, Tongaat.

BECKSTROM-STERNBERG, S.M., DUKE, J.A. and WAIN, K.K. 1994. The ethnobotany database. Genome Informatics Group, National Agricultural Library. U.S. Department of Agriculture, Beltsville, MD (USA).

BENTLEY, K.W. 1986. ß-Phenylethylamines and the isoquinoline alkaloids. *Natural Product Reports* 3 (1986): 153-169.

BERGOÑÓN, S., CODINA, C., BASTIDA, J., VILADOMAT, F. and MELÉ, E. 1992. The shake liquid culture as an alternative way to the multiplication of *Narcissus* plants. *Acta Horticulturae* 325: 447-452.

BERGOÑÓN, S., CODINA, C., BASTIDA, J., VILADOMAT, F. and MELÉ, E. 1996. Galanthamine production in "shoot-clump" cultures of *Narcissus confusus* in liquid-shake medium. *Plant Cell, Tissue and Organ Culture* 45: 191-199.

BERUTO, M. and DEBERGH, P. 1992. Somatic embryogenesis in *Ranunculus asiaticus* L. hybr. thalamus cultivated *in vitro*. *Plant Cell, Tissue and Organ Culture* 29: 161-165.

BEUTNER, D. and FRAHM, A.W. 1986. New alkaloids from *Crinum asiaticum*. *Plant Medica* 52:523.

BIZIMANA, N. 1994. Traditional veterinary practice in Africa. Deutsche Gesellschaft für Technicsche Zusammenarbeit (GIZ) GmbH, Eschborn (Germany). ISBN 3-88085-502-1.

BLACKER, M. 1994. Bulbs for all climates. ACP Publishing, Sydney. ISBN 1-86396-024-4.

BONNER, J. 1995. Flower bulbs slow brain disease. *New Scientist*, 11 February 1995, p.21.

BONNIER, F.J.M. and VAN TUYL, J.M. 1997. Long term *in vitro* storage of lily: Effects of temperature and concentration of nutrients and sucrose. *Plant Cell, Tissue and Organ Culture* 49: 81-87.

BOONEKAMP, P.M. 1997. The role of external factors in growth and development of flower bulbs and bulb-flowers: An update since 1992. *Acta Horticulturae* 430: 35-43.

BRISTOW, D. 1988. The natural history of southern Africa. Struik, Cape Town. ISBN 0-86977-737-8.

BRYAN, J. E. 1989. Bulbs. Volume II. A-H. Timber Press, Portland. ISBN 07470-0231-2.

BRYANT, A.T. 1905. A Zulu-English dictionary. Marianhill Mission Press, Pinetown.

BRYANT, A.T. 1966. *Zulu medicine and medicine-men.* Struik, Cape Town. ISBN 0-908379-44-7.

BURBIDGE, A. 1994. Secondary plant metabolites from tissue culture. In: Hunter, C.F. (Ed). *In vitro* cultivation of plant cells. Butterworth-Heinemann, Oxford. ISBN 0-7506-0554-5.

BUTCHER, D.N. 1977. Secondary products in tissue cultures. In: Reinert, J. and Bajaj, Y.P.S. (Eds). Applied and fundamental aspects of plant cell, tissue and organ culture. Springer-Verlag, Berlin. ISBN 3-540-07677-8.

CHATTOPADHYAY, S., CHATTOPADHYAY, U., MATHUR, P.P., SAINI, K.S. and GHOSAL, S. 1983. Effects of hippadine, an Amaryllidaceae alkaloid, on testicular function in rats. *Plant Medica* 49(4): 252-254.

CHAUMONT, J.P., DE SCHEEMAEKER, H. and ROUSSEAU, J. 1978. Antifungal properties of some members of the Amaryllidaceae. *Plantes Medicinales et Phytotherapie* 12(2): 157-161.

CHITTENDEN, F.J. 1956. The Royal Horticultural Society dictionary of gardening. A practical and scientific encyclopaedia of horticulture. Clarendon Press, Oxford.

CHOW, Y.N., SELBY, C. and HARVEY, B.M.R. 1992[a]. Stimulation by sucrose of *Narcissus* bulbil formation *in vitro*. *Journal of Horticultural Science* 67(2): 289-293.

CHOW, Y.N., SELBY, C. and HARVEY, B.M.R. 1992[b]. A simple method for maintaining high multiplication of *Narcissus* shoot cultures *in vitro*. *Plant Cell, Tissue and Organ Culture* 30: 227-230.

CHOW, Y.N., SELBY, C., FRASER, T.W. and HARVEY, B.M.R. 1993. Basal plate tissue in *Narcissus* bulbs and in shoot clump cultures: Its structure and role in organogenic potential of single leaf cultures. *Annals of Botany* 71: 437-443.

CHURIKOVA, O.A. and BARYKINA, R.P. 1995. Regeneration ability of some bulbous and cormous monocotyledons *in vitro*. Morphogenetic aspect. *Moscow University Biological Sciences Bulletin* 50(2): 50-56.

COLLINGE, M.A. and YEOMAN, M.M. 1986. The relationship between tropane alkaloid production and structural differentiation in plant cell cultures of *Atropa belladonna* and *Hyoscyamus muticus*. In: Morris, P., Scragg, A.H., Stafford, A. and Fowler, M.W. (Eds). Secondary metabolism in plant cell cultures. Cambridge University Press, Cambridge. ISBN 0 521 32889 6.

COOK, J.W. and LOUDON, J.D. 1954. Alkaloids of the Amaryllidaceae. In: Manske, R.H.F. and Holmes, H.L. (Eds). The alkaloids: Chemistry and physiology. Volume II. Academic Press, New York, pp. 331-337.

CORDELL, G.A. 1981. Introduction to alkaloids: A biogenetic approach. John Wiley & Sons, New York. ISBN 0-471-03478-9.

COTTON, C.M. 1996. Ethnobotany. Principles and applications. John Wiley & Sons, Chichester. ISBN 0-471-95537-X.

COUILLEROT, E., CARON, C., AUDRAN, J-C., JARDILLIER, J-C. and CHÉNIEUX, J-C. 1996. Furoquinoline alkaloid accumulation in *Fagara zanthoxyloides* cell cultures is highly dependent on the presence of exogenous benzylaminopurine. *Plant Growth Regulation* 19: 203-206.

COX, P.A. and BALICK, M.J. 1994. The ethnobotanical approach to drug discovery. *Scientific American* 270(6): 60-65.

CRAIB, C. 1997. *Crinum minimum*: One of South Africa's most unusual bulbous plants. *Herbertia* 52: 101-108.

CRAIB, C. and BLACKMORE, A. 1997. *Crinum acaule* in Zululand, South Africa. *Herbertia* 52: 72-80.

CRISP, P. and WALKEY, D.G.A. 1974. The use of aseptic meristem culture in cauliflower breeding. *Euphytica* 23: 305-313.

CUNNINGHAM, T. 1984. Medicinal plants and witchdoctors. Are we barking up the wrong tree? *African Wildlife* 38(6): 247-249.

CUNNINGHAM, A.B. 1988. An investigation of the herbal medicine trade in Natal / Kwazulu. Investigational Report No. 29. University of Natal, Institute of Natural Resources, Pietermaritzburg.

CUNNINGHAM, A.B. 1990. African medicinal plants: Setting priorities at the interface between conservation and primary health care. University of Natal, Institute of Natural Resources, Pietermaritzburg.

CUNNINGHAM, A.B. 1991. The herbal medicine trade. Resource depletion and environmental management for a hidden economy. In: Preston-Whyte, E. and Rogerson, C. (Eds). South Africa's informal economy. Oxford University Press, Cape Town. ISBN 0-19-570633-1.

CUNNINGHAM, A.B. and ZONDI, A.S. 1991. Cattle owners and traditional medicines for livestock. Investigational Report No. 69. University of Natal, Institute of Natural Resources, Pietermaritzburg.

CUNNINGHAM, A.B., DE JAGER, P.J. and HANSEN, L.C.B. 1993. The indigenous plant use programme. 1992. Foundation for Research Development, South Africa. ISBN 1-874916-03-9.

CUSTERS, J.B.M. and BERGERVOET, J.H.W. 1992. Differences between *Nerine* hybrids in micropropagation potential. *Scientia Horticulturae* 52: 247-256.

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

DEBERGH, P.C.A. 1994. The *in vitro* techniques: Their contribution to breeding and multiplication of ornamentals. *Acta Horticulturae* 353: 122-133.

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

DEBERGH, P.C. and ZIMMERMAN, R.H. 1991. Micropropagation: Technology and application. Kluwer Academic Publishers, Dordrecht. 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.

DE BRUYN, M.H., FERREIRA, D.I., SLABBERT, M.M. and PRETORIUS, J. 1992. *In vitro* propagation of *Amaryllis belladonna*. *Plant Cell, Tissue and Organ Culture* 31: 179-184.

DECENDIT, A., LIU, D., OUELHAZI, L., DOIREAU, P., MÉRILLON, J-M. and RIDEAU, M. 1992. Cytokinin-enhanced accumulation of indole alkaloids in *Catharanthus roseus* cell cultures - The factors affecting the cytokinin response. *Plant Cell Reports* 11: 400-403.

DE HERTOGH, A.A. and LE NARD, M. 1993. The physiology of flower bulbs. A comprehensive treatise on the physiology and utilization of ornamental flowering bulbous and tuberous plants. Elsevier, Amsterdam. ISBN 0-444-87498-4.

DEMEYER, K., VANHASTE, H., VAN DER VELDE, H. and DEJAEGERE, R. 1992. Introductory study for the optimization of growth and alkaloid production by cell cultures of *Datura stramonium* L. *Acta Horticulturae* 306: 210-218.

DESAMERO, N.V., ADELBERG, J.W., HALE, A., YOUNG, R.E. and RHODES, B.B. 1993. Nutrient utilization in liquid / membrane system for watermelon micropropagation. *Plant Cell, Tissue and Organ Culture* 33: 265-271.

DE VROOMEN, C.O.N. 1995. Somatic embryogenesis a revolution? A study in the ornamental flower bulb sector. *Mededeling Landbouw Economisch Instituut*. No. 546.

DICOSMO, F. and MISAWA, M. 1995. Plant cell and tissue culture: Alternatives for metabolite production. *Biotechnology Advances* 13(3): 425-453.

DODDS, J.H. 1991. *In vitro* methods for conservation of plant genetic resources. Chapman & Hall, London. ISBN 0-412-33870-X.

DREWES, F.E. and VAN STADEN, J. 1994. *In vitro* propagation of *Gethyllis linearis* L.Bol., a rare indigenous bulb. *South African Journal of Botany* 60(5): 295-296.

DUNCAN, G. 1982. *Crinum nerinoides* Bak. - a dwarf species from South West Africa. *Veld & Flora* 3(1): 19.

DUNSTAN, D.I. and SHORT, K.C. 1977. *In vitro* studies on organogenesis and growth in *Allium cepa* tissue cultures. *Acta Horticulturae* 78: 139-148.

DU PLESSIS, N. and DUNCAN, G. 1989. Bulbous plants of Southern Africa. A guide to their cultivation and propagation. Tafelberg, Cape Town. ISBN 0-624-02659-0.

DURI, Z.J., SCOVILL, J.P. and HUGGINS, J.W. 1994. Activity of a methanolic extract of Zimbabwean *Crinum macowanii* against exotic RNA viruses *in vitro*. *Phytotherapy Research* 8: 121-122.

EICHHORN, J., TAKADA, T., KITA, Y. and ZENK, M. 1998. Biosynthesis of the Amaryllidaceae alkaloid galanthamine. *Phytochemistry* 49(4): 1037-1047.

EL-BAHR, M.K., HUSSEIN, M.S. and MOURSY, H.A. 1989. Effect of some growth regulators on the growth and alkaloid production of *Datura stramonium* L. cultured in vitro. *Egyptian Journal of Botany* 32(1-2): 53-62.

ELGORASHI, E.E. 2000. Alkaloids from three South African *Crinum* species. PhD thesis, University of Natal, Pietermaritzburg.

ELGORASHI, E.E., DREWES, S.E. and VAN STADEN, J. 1999. Alkaloids from *Crinum bulbispermum*. *Phytochemistry* 52: 533-536.

ELGORASHI, E.E., DREWES, S.E. and VAN STADEN, J. 2001[a]. Alkaloids from *Crinum moorei*. *Phytochemistry* 56: 637-640.

ELGORASHI, E.E., DREWES, S.E. and VAN STADEN, J. 2001[b]. Alkaloids from *Crinum macowanii*. *Biochemical Systematics and Ecology* 29: 749-750.

ELGORASHI, E.E. and VAN STADEN, J. 2001. Alkaloids from *Crinum lugardiae*. *Biochemical Systematics and Ecology* 29: 751-752.

EL-HAFIZ, M.A.A., RAMADAN, M.A. and ANTON, R. 1990. Minor phenolic constituents of *Crinum augustum*. *Journal of Natural Products* 53(5): 1349-1352.

ELIOVSON, S. 1967. Bulbs for the gardener. Howard Timmins, Cape Town.

ELIOVSON, S. 1980. Wild flowers of southern Africa. All-in-one guide to shrubs, trees, succulents, bulbs, annuals, perennials; how to grow and identify them. M. ISBN 0-86954-088-2.

EMANOIL, M. 1994. Encyclopedia of endangered species. Gale Research Inc., Detroit. ISBN 0-8103-8857.

ETIENNE, E., TEISSON, C., ALVARD, D., LARTAUD, M., BERTHOULY, M., GEORGET, F., ESCALONA, M. and LORENZO, J.C. 1999. Temporary immersion for plant tissue culture. In: Altman, A., Ziv, M. and Izhar, S. (Eds). Plant biotechnology and *in vitro* biology in the 21st century. Kluwer Academic Publishers, The Netherlands, pp. 629-632.

ETIENNE-BARRY, D., BERTRAND, B., VASQUEZ, N. and ETIENNE, H. 1999. Direct sowing of *Coffee arabica* somatic embryos mass-produced in a bioreactor and regeneration of plants. *Plant Cell Reports* 19: 111-117.

ETKIN, N. 1986. Plants in indigenous medicine and diet. Biobehavioural approaches. Redgrave Publishing Company, Bedford Hills, New York. ISBN 0-913178-02-0.

EVERETT, T.H. 1981. The New York Botanical Garden illustrated encyclopaedia of horticulture. Garland Publishing, New York, pp. 916-918. ISBN 0-8240-7233-2.

FANGAN, B.M. and NORDAL, I. 1993. A comparative analysis of morphology, chloroplast-DNA and distribution within the genus *Crinum* (Amaryllidaceae). *Journal of Biogeography* 20:55-61.

FARNSWORTH, N.R. and SOEJARTO, D.D. 1991. Global importance of medicinal plants. In: Akerele, O., Heywood, V. and Synge, H. (Eds). Conservation of medicinal plants. Cambridge University Press, Cambridge. ISBN 0-521-39206-3.

FENNELL, C.W. and VAN STADEN, J. 2001. *Crinum* species in traditional and modern medicine. *Journal of Ethnopharmacology* 78: 15-26.

FERNANDO, A., TOMBOLATO, C., AZEVEDO, C. and NAGAI, V. 1994. Effects of auxin treatment on *in vivo* propagation of *Hippeastrum hybridum* Hort. by twin-scaling. *HortScience* 29: 922.

FINNIE, J.F. 1988. Tissue culture of selected indigenous monocotyledons. Ph.D. thesis. University of Natal, Pietermaritzburg.

FOUNTAIN, W.M. and O'ROURKE, E.N. 1980. Investigations of *in vitro* culture of *Hippeastrum* as a method of rapid propagation. *HortScience* 15(3): 274-275.

FOWLER, M.W. 1983. Commercial applications and economic aspects of mass plant cell culture. In: Mantell, S.H. and Smith, H. (Eds). Plant Biotechnology. Cambridge University Press, Cambridge, pp. 75-108. ISBN 0-521-24550.

FOWLER, M.W. 1986. Biotechnological applications of plant tissue cultures. In: Alani, D.I. and Moo-Young, M. (Eds). Perspectives in biotechnology and applied microbiology. Arab Gulf conference on biotechnology and applied microbiology, Riyadh (Saudi Arabia), 12-15 November 1984, pp. 295-316. ISBN 1-85166-055-0.

FRAHMY, F.G. and MOHAMED, M.S. 1992. Buphanisine as a new plant antiviral agent. *Assiut Journal of Agricultural Sciences* 20: 279-292.

FRIDBORG, G. and ERIKSSON, T. 1975. Effects of activated charcoal on growth and morphogenesis in cell cultures. *Physiologia Plantarum* 34: 306-308.

FUGANTI, C. 1975. The Amaryllidaceae alkaloids. In: Manske, R.H.F. (Ed). The alkaloids. Chemistry and physiology. Volume XV. Academic Press, New York, pp. 83-164.

GELFAND, M., MAV, S., DRUMMOND, R.B. and NDEMERA, B. 1985. The traditional medicinal practitioner in Zimbabwe. His principles of practice and pharmacopoeia. Mambo Press, Gweru. ISBN 0-86922-350-X.

GEORGE, E.F. 1993. Plant propagation by tissue culture. Part 1. The technology. Exegetics Limited, Edington. ISBN 0-9509325-4-X.

GHOSAL, S., KUMAR, Y. and SINGH, S. 1984. Glucosyloxy alkaloids from *Pancratium biflorum*. Phytochemistry 23(5): 1167-1171.

GHOSAL, S., SAINI, K.S. and RAZDAN, S. 1985. *Crinum* alkaloids. Their chemistry and biology. *Phytochemistry* 24(10): 2141-2156.

GHOSAL, S., SINGH, S.K. and UNNIKRISHNAN, S.G. 1990. Effects of stress on alkaloid metabolism in *Crinum asiaticum*. *Phytochemistry* 29(3): 805-811.

GILAD, Z. and BOROCHOV, A. 1993. Hot-water treatment of *Liatris* tubers. *Scientia Horticulturae* 56: 61-69.

GINZBURG, C. and ZIV, M. 1973. Hormonal regulation of cormel formation in *Gladiolus* stolons grown *in vitro*. *Annals of Botany* 37: 219-224.

GITHENS, T.S. 1949. Drug plants of Africa. University of Pennsylvania Press, Philadelphia.

GIULIETTI, A.M. and ERTOLA, R.J. 1999. Biotechnological strategies for production of plants and secondary metabolites of pharmaceutical interest. *Acta Horticulturae* 502: 269-279.

GLASBY, J.S. 1991. Dictionary of plants containing secondary metabolites. Taylor & Francis, London. ISBN 0-85066-423-3.

GONZALEZ-BENITO, M.E. and ALDERSON, P.G. 1992. Callus induction and plant regeneration in *Alstroemeria*. *Journal of Experimental Botany* 43: 205-211.

GORINOVA, N.I., ATANASSOV, A.I., STOJANOV, D.V. and TENCHEVA, J. 1993. Influence of chemical composition of soils on the galanthamine content in *Leucojum aestivum*. *Journal of Plant Nutrition* 16(9): 1631-1636.

GREENBLATT, H.M., KRYGER, G., LEWIS, T., SILMAN, I. and SUSSMAN, J.L. 1999. Structure of acetylcholinesterase complexed with (-)-galanthamine at 2.3 Å resolution. *FEBS Letters* 463: 321-326.

GROOTAARTS, H., SCHEL, J.H.N. and PIERIK, R.L.M. 1981. The origin of bulblets formed on excised twin-scales of *Nerine bowdenii* W.Watts. *Plant Cell, Tissue and Organ Culture* 1: 39-46.

GRUNDON, M.F. 1984. Amaryllidaceae alkaloids. *Natural Product Reports* 1: 247-250.

GRUNDON, M.F. 1985. Amaryllidaceae alkaloids. *Natural Product Reports* 2: 247-251.

GRUNDON, M.F. 1987. Amaryllidaceae alkaloids. *Natural Product Reports* 4: 89-94.

GRUNDON, M.F. 1989. Amaryllidaceae alkaloids. *Natural Product Reports* 6: 79-84.

GUDE, H. and DIJKEMA, M.H.G.E. 1997. Somatic embryogenesis in tulip. *Acta Horticulturae* 430: 275-280.

GUNDIDZA, M. 1986. Screening extracts from Zimbabwean higher plants. II. Antifungal properties. *Fitoterapia* 57: 111-114.

HALL, J.B. and TOMLINSON, P.B. 1973. Natural callus-like tissue produces vegetative buds on the leaves of the monocotyledon *Haemanthus cinnabrinus* Decaisne (Amaryllidaceae). *Nature (London). New Biology* 243: 252-255.

HALL, A.V., DE WINTER, M., DE WINTER, B. and VAN OOSTERHOUT, S.A.M. 1980. Threatened plants of southern Africa. CSIR, Cooperative Scientific Programmes, Pretoria. ISBN 0-7988-1780-1.

HAMILTON, A. 1993. Conservation and medicinal plants: Present utilization and future products. *Acta Horticulturae* 330: 43-49.

HANKS, G.R. 1993. *Narcissus*. In: De Hertogh, A. and Le Nard, M. (Eds). The physiology of flower bulbs. Elsevier Science, Amsterdam, pp. 463-558. ISBN 0-444-87498-4.

HANKS, G.R. and REES, A.R. 1979. Twin-scale propagation of *Narcissus*: A review. *Scientia Horticulturae* 10: 1-14.

HANKS, G.R., SHAIK, G. and JONES, S.K. 1986. Bulbil production in *Narcissus*: The effect of temperature and duration of storage on bulb unit development and subsequent propagation by twin-scaling. *Annals of Applied Biology* 109: 417-425.

HANNWEG, K., WATT, M.P. and BERJAK, P. 1996. A simple method for the micropropagation of *Bowiea volubilis* from inflorescence explants. *Botanical Bulletin of Academia Sinica* 37: 213-218.

HARA, M., KITAMURA, T., FUKUI, H. and TABATA, M. 1993. Induction of berberine biosynthesis by cytokinins in *Thalictrum minus* suspension cultures. *Plant Cell Reports* 12(2): 70-73.

HARBORNE, J.B. and BAXTER, H. 1993. Phytochemical dictionary. A handbook of bioactive compounds from plants. Taylor & Francis, London. ISBN 0-85066-736-4.

HAUPTMANN, R.M., WIDHOLM, J.M. and PAXTON, J.D. 1985. Benomyl: A broad spectrum fungicide for use in plant cell and protoplast culture. *Plant Cell Reports* 4: 129-132.

HEDBERG, I. 1979. Possibilities and needs for conservation of plant species and vegetation in Africa. In: Hedberg, I. Systematic botany, plant utilization and biosphere conservation. Proceedings of a symposium held in Uppsala in commemoration of the 500th anniversary of the University. Almqvist and Wiksell, Stockholm. ISBN 91-22-00177-8.

HEINZ, H.J. and MAGUIRE, B. The ethno-biology of the *!kõ* Bushmen: Their ethnobotanical knowledge and plant lore. Occasional Paper No. 1. Botswana Society, Gaborone.

HILTON-TAYLOR, C. 1996. Red data list of Southern African plants. Strelitzia 4. National Botanical Institute, Pretoria. ISBN 1-87490-729-3.

HOBBS, M.C. and YEOMAN, M.M. 1991. Effect of light on alkaloid accumulation in cell cultures of *Nicotiana* species. *Journal of Experimental Botany* 42(244): 1371-1378.

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.

HOSHINO, O. 1998. The Amaryllidaceae alkaloids. In: Cordell, G.A. (Ed). The Alkaloids: Chemistry and biology. Volume 51. Academic Press, p. 324. ISBN 0124695515.

HOSOKI, T. and ASAHIRA, T. 1980. *In vitro* propagation of *Narcissus*. *HortScience* 15(5): 602-603.

HUANG, C.W., OKUBO, H. and UEMOTO, S. 1990. Importance of two scales in propagating *Hippeastrum hybridum* by twin-scaling. *Scientia Horticulturae* 42: 141-149.

HUGHES, K.W. 1981. Ornamental species. In: Conger, B.V. (Ed). Cloning agricultural plants via *in vitro* techniques. CRC Press, Boca Raton, pp. 5-50. ISBN 0-8493-5797-7.

HUSSEY, G. 1975. Totipotency in tissue explants and callus of some members of the Liliaceae, Iridaceae and Amaryllidaceae. *Journal of Experimental Botany* 26: 253-262.

HUSSEY, G. 1976[a]. *In vitro* release of axillary shoots from apical dominance in monocotyledonous plantlets. *Annals of Botany* 40(2): 1323-1325.

HUSSEY, G. 1976[b]. Plantlet regeneration from callus and parent tissue in *Ornithogalum thyrsoides*. *Journal of Experimental Botany* 27: 375-382.

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

HUSSEY, G. 1978. The application of tissue culture to the vegetative propagation of plants. *Science Progress, Oxford* 65: 185-208.

HUSSEY, G. 1980. Propagation of some members of the Liliaceae, Iridaceae and Amaryllidaceae by tissue culture. In: Brickell, C.D., Cutler, D.F. and Gregory, M. (Eds). Petaloid monocotyledons: Horticultural and botanical research. Papers presented at a symposium; Research into monocotyledons of horticultural importance; Organized by the Linnanean Society of London and the Royal Horticultural Society, London 3-5 April 1979. Academic Press, London, pp. 33-42. ISBN 0-12-133950-5.

HUSSEY, G. 1982[a]. *In vitro* propagation of monocotyledonous bulbs and corms. In: Fujiwara, A. (Ed). Plant tissue culture. 1982. Proceedings of the 5th International Congress of Plant Tissue and Cell Culture. Japanese Association for Plant Tissue Culture, Tokyo, pp. 677-680.

HUSSEY, G. 1982[b]. *In vitro* propagation of *Narcissus*. *Annals of Botany* 49: 707-719.

HUSSEY, G. 1986. Problems and prospects in the *in vitro* propagation of herbaceous plants. In: Withers, L.A. and Alderson, P.G. (Eds). Plant tissue culture and its agricultural applications. Butterworth, London, pp.69-84. ISBN 0-407-00921-3.

HUSSEY, G. and STACEY, N.J. 1981. *In vitro* propagation of potato (*Solanum tuberosum* L.). *Annals of Botany* 48: 787-796.

HUTCHINGS, A. 1989. A survey and analysis of traditional medicinal plants as used by the Zulu, Xhosa and Sotho. *Bothalia* 19: 111-123.

HUTCHINGS, A. and JOHNSON, C. 1986. Glimpses of a Xhosa herbal. *Veld & Flora* 72(2): 59-62.

HUTCHINGS, A., SCOTT, A.H., LEWIS, G. and CUNNINGHAM, A.1996. Zulu medicinal plants. An inventory. University of Natal Press, Scottsville (Pietermaritzburg). ISBN 0-86980-893-1.

HUXLEY, A. 1984. Green inheritance. The World Wildlife Fund book of plants. Collins Harvill, London. ISBN 0-00-272614-9.

HUXLEY, A., GRIFFITHS, M. and LEVY, M. 1992. The new Royal Horticultural Society dictionary of gardening. Macmillan, London. ISBN 0-333-47494-5.

HYANGYOUNG, J., BONGKYU, P. and CHANGJAE, Y. 1997. Effect of growth regulators, chilling treatment and light on adventitious shoots differentiation by *in vitro* scape culture of *Narcissus pseudo-narcissus* L. *RDA Journal of Horticultural Science* 39(1): 82-88.

IEVEN, M., VAN DEN BERGHE, D.A. and VLIETINCK, A.J. 1983. Plant antiviral agents. IV. Influence of lycorine on growth pattern of three animal viruses. *Planta Medica* 49(2): 109-114.

ILAN, A., ZIV, M. and HALEVY, A.H. 1995. Propagation and corm development of *Brodiaea* in liquid cultures. *Scientia Horticulturae* 63: 101-112.

IQBAL, S.H. and BAREEN, F. 1986. Mycorrhizae of the Liliflorae. I. Vesicular-arbuscular mycorrhizal infections in outer dried sheathing leaves from bulbs of some ornamental lilies. *Biologia - Pak.* 32(2): 323-328.

ISAAC, W.M.E. and McGILLIVRAY, G. 1965. A note on the water-storing seeds of two species of *Crinum* and some other South African Amaryllidaceae (sub-family Amaryllidoiodeae). *Annals of Botany, N.S.* 29: 739-740.

JACOBS, G., RICHARD, M., ALLDERMAN, L.A. and THERON, K.I. 1992. Direct and indirect organogenesis in tissue cultures of *Nerine bowdenii* W.Watts. *Acta Horticulturae* 325: 475-479.

JACOT GUILLARMOD, A. J. 1971. Flora of Lesotho (Basutoland). Cramer, Lehre.

JANSEN VAN VUUREN, P.J., COETZEE, J. and COERTSE, A. 1993. South African flowering plants with a potential as future floriculture crops. *Acta Horticulturae* 337: 65-71.

JHA, S., SAHU, N.P. and MAHATO, S.B. 1991. Callus induction, organogenesis and somatic embryogenesis in three chromosomal races of *Urginea indica* and production of bufadienolides. *Plant Cell, Tissue and Organ Culture* 25: 85-90.

JOHN, E.V.O. 1961. The alkaloids of the Amaryllidaceae. The alkaloids of *Crinum longiflorum*. The structure of Buphanitine. The synthesis of a possible degradation product of Albomaculine. PhD thesis, Department of Chemistry and Chemical Engineering, University of Natal, Pietermaritzburg.

KALAYCIOGLU, A. and ONER, C. 1994. Investigation of the antimutagenic effect of some plant extracts with Ames-Salmonella test system. *Turkish Journal of Botany* 18(3): 117-122.

KIM, D-I., PEDERSEN, H. and CHIN, C-K. 1988. Effects of light on berberine production in cell suspension cultures of *Thalictrum rugosum*. *Biotechnology Letters* 10(10): 709-712.

KIM, K. and DE HERTOGH, A.A. 1997. Tissue culture of ornamental flowering bulbs (geophytes). *Horticultural Reviews* 18: 87-169.

KIVIHARJU, E., TUOMINEN, U. and TÖRMÄLÄ, T. 1992. The effect of explant material on somatic embryogenesis of *Cyclamen persicum* Mill. *Plant Cell, Tissue and Organ Culture* 28: 187-194.

KOOPOWITZ, H. 1986. Conservation problems in the Amaryllidaceae. *Herbertia* 42: 21-25.

KOOPOWITZ, H. and KAYE, H. 1983. Plant extinction: a global crisis. Stone Wall Press, Washington. ISBN 0-913276-44-8.

KOZAK, D. 1991. Shoots regeneration from various parts of *Narcissus* cv. Carlton through tissue culture. *Prace Instytutu Sadownictwa i Kwiaciarstwa w Skierniewicach. Seria B, Rosliny Ozdobne* 16: 41-48.

KRIKORIAN, A.D. and KANN, R.P. 1981. Plantlet production from morphogenetically competent cell suspensions of Daylily. *Annals of Botany* 47: 679-686.

KRIKORIAN, A.D. and KANN, R.P. 1986. Regeneration in Liliaceae, Iridaceae and Amaryllidaceae. In: Vasil, I.K. (Ed). Cell culture and somatic cell genetics of plants. Volume 3. Plant regeneration and genetic variability. Academic Press, Orlando, pp. 187-205. ISBN 0-12-715003-X.

KRITZINGER, E.M., JANSEN VAN VUUREN, R., WOODWARD, B., RONG, I.H., SPREETH, M.H. and SLABBERT, M.M. 1998. Elimination of external and internal contaminants in rhizomes of *Zantedeschia aethiopica* with commercial fungicides and antibiotics. *Plant Cell, Tissue and Organ Culture* 52: 61-65.

KROMER, K.D. 1985. Regeneration of some monocotyledonous plants from subterranean organs *in vitro*. *Acta Agrobotanica* 38(2): 65-87.

KUKULCZANKA, K. and KROMER, K. 1988. Propagation of *Vallota purpurea* Herb. through tissue culture. *Acta Horticulturae* 226: 129-135.

KURATA, H., SEKI, M. and FURUSAKI, S. 1994. Light effect to promote secondary metabolite production of plant cell culture. In: Furusaki, S. (Ed). Advances in plant biotechnology. Studies in plant science. 4. Elsevier, Amsterdam, pp. 103-133.

KUTNEY, J.P. 1995. Plant cell culture combined with chemistry - potentially powerful routes to clinically important compounds. In: Terzi, M., Cella, R. and Falavigna, A. (Eds). Current issues in plant molecular and cellular biology. Kluwer Academic Publishers, Dordrecht. ISBN 0-7923-3322-5.

LANGENS-GERRITS, M. and NASHIMOTO, S. 1997. Improved protocol for the propagation of *Narcissus in vitro*. *Acta Horticulturae* 430: 311-313.

LANGENS-GERRITS, M., LILIEN-KIPNIS, H., CROES, T., MILLER, W., KOLLÖFFEL, C. and DE KLERK, G.J. 1997. Bulb growth in Lily regenerated *in vitro*. *Acta Horticulturae* 430: 267-273.

LANGENS-GERRITS, M., ALBERS, M. and DE KLERK, G. 1998. Hot-water treatment before tissue culture reduces initial contamination in *Lilium* and *Acer*. *Plant Cell, Tissue and Organ Culture* 52: 75-77.

LEHMILLER, D.J. 1987. A South African Crinum safari. *Herbertia* 43(2): 50-59.

LEHMILLER, D.J. 1996. Cultivation of African *Crinum* in pots and tubs. *Herbertia* 51: 33-37.

LEIFFERT, C. and WAITES, W.M. 1994. Dealing with microbial contaminants in plant tissue and cell culture. Hazard analysis and critical control points. In: Lumsden. P.J., Nicholas, J.R. and Davies, W.J. (Eds). Physiology, growth and development of plants in culture. Kluwer, Dordrecht, pp. 363-378. ISBN 0-7923-2516-8.

LE MAOUT, E. and DECAISNE, J. 1873. A general system of botany: Descriptive and analytical, in two parts. Longmans, Green, London, p. 789.

LE NARD, M. and CHANTELOUBE, F.I. 1992. *In vitro* culture of explants excised from growing stems of tulip (*Tulipa gesneriana* L.): Problems related to bud and bulblet formation. *Acta Horticulturae* 325: 435-440.

LE NARD, M. and DE HERTOGH, A.A. 1993. Research perspectives for flower bulbs. In: De Hertogh, A.A. and Le Nard, M. (Eds). The physiology of flower bulbs. Elsevier, Amsterdam, pp. 777-782. ISBN 0-444-87498-4.

LESHEM, B., LILIEN-KIPNIS, H. and STEINITZ, B. 1982. The effect of light and of explant orientation on the regeneration and subsequent growth of bulblets on *Lilium longiflorum* Thunb. bulb-scale sections cultured *in vitro*. *Scientia Horticulturae* 17: 129-136.

LESZCZYNSKA-BORYS, H. 1995. Ornamental plants in the customs of the Sierra Norte of Puebla. *Acta Horticulturae* 391: 251-260.

LEVIN, R., GABA, V., TAL, B., HIRSCH, S., DENOLA, D. and VASIL, I.K. 1988. Automated plant tissue culture for mass propagation. *Biotechnology* 6: 1035-1040.

LEWIS, J.R. 1990. Amaryllidaceae alkaloids. *Natural Product Reports* 7: 549-556.

LEWIS, J.R. 1992. Amaryllidaceae and *Sceletium* alkaloids. *Natural Product Reports* 9: 183-191.

LEWIS, J.R. 1993. Amaryllidaceae and *Sceletium* alkaloids. *Natural Product Reports* 10: 291-299.

LEWIS, J.R. 1994. Amaryllidaceae and *Sceletium* alkaloids. *Natural Product Reports* 11: 329-332.

LEWIS, J.R. 1996. Amaryllidaceae and *Sceletium* alkaloids. *Natural Product Reports* 13: 171-176.

LEWIS, J.R. 1997. Amaryllidaceae alkaloids. *Natural Product Reports* 14: 303-308.

LEWIS, J.R. 1998. Amaryllidaceae and *Sceletium* alkaloids. *Natural Product Reports* 15(1): 107-110.

LEWIS, J.R. 1999. Miscellaneous alkaloids: Amaryllidaceae, *Sceletium*, muscarine, imidazole, oxazole, peptide and other miscellaneous alkaloids. *Natural Product Reports* 16: 389-416.

LEWIS, J.R. 2000. Amaryllidaceae, muscarine, imidazole, oxazole, thiazole and peptide alkaloids, and other miscellaneous alkaloids. *Natural Product Reports* 17: 57-84.

LIKHITWITAYAWUID, K., ANGERHOFER, C.K., CHAI, H.Y., PEZZUTO, J.M., CORDELL, G.A. and RUANGRUNGSI, N. 1993. Cytotoxic and antimalarial alkaloids from the bulbs of *Crinum amabile*. *Journal of Natural Products* 56(8): 1331-1338.

LILIEN-KIPNIS, H., ZIV, M. and KAHANY, S. 1990. Proliferation and plantlet regeneration from inflorescence derived *Nerine* explants cultured *in vitro*. In: De Jong, J. (Ed). Integration of *in vitro* techniques in ornamental plant breeding. Proceedings, Symposium, 10-14 November 1990. EUCARPIA, Wageningen, pp. 28-33.

LILIEN-KIPNIS, H., ZIV, M., KAHANY, S. and AZIZBEKOV, N. 1992. Proliferation and regeneration of *Nerine* in liquid culture. *Acta Horticulturae* 325: 467-473.

LILIEN-KIPNIS, H., AZIZBEKOVA, N. and ZIV, M. 1994. Scaled-up proliferation and regeneration of *Nerine* in liquid cultures. II. Ontogeny of somatic embryos and bulblet regeneration. *Plant Cell, Tissue and Organ Culture* 39: 117-123.

LIN, H., DE JEU, M.J. and JACOBSEN, E. 1997. Direct shoot regeneration from excised leaf explants of *in vitro* grown seedlings of *Alstroemeria* L. *Plant Cell Reports* 16: 770-774.

LIN, H., DE JEU, M.J. and JACOBSEN, E. 1998. Formation of shoots from leaf axils of *Alstroemeria*: The effect of the position on the stem. *Plant Cell, Tissue and Organ Culture*. 52: 165-169.

LIN, H-S., VAN DER TOORN, C., RAEMAKERS, K.J.J.M., VISSER, R.G.F., DE JEU, M.J. and JACOBSEN, E. 2000. Development of a plant regeneration system based on friable embryogenic callus in the ornamental *Alstroemeria*. *Plant Cell Reports* 19: 529-534.

LUMSDEN, P.J., PRYCE, S. and LEIFERT, C. 1990. Effect of mineral nutrition on the growth and multiplication of *in vitro* cultured plants. In: Nijkamp, H.J.J., van der Plas, L.H.W. and van Aartrijk, J. (Eds). Current plant science and biotechnology in agriculture. Progress in plant cellular and molecular biology. Kluwer Academic Publishers, Dordrecht, The Netherlands, pp. 108-113. ISBN 0-7923-0873-5.

McALISTER, B.G., JÄGER, A.K. and VAN STADEN, J. 1998[a]. Micropropagation of *Babiana* spp. *South African Journal of Botany* 64(1): 88-90.

McALISTER, B.G., STRYDOM, A. and VAN STADEN, J. 1998[b]. *In vitro* propagation of some *Cyrtanthus* species. *South African Journal of Botany* 64(3): 228-231.

McCARTAN, S.A. and VAN STADEN, J. 1998. Micropropagation of the medicinal plant, *Scilla natalensis* Planch. *Plant Growth Regulation* 25: 177-180.

McCRACKEN, D.P. and McCRACKEN, P.A. 1990. Natal: The garden colony. Frandsen Publishers, Sandton. ISBN 0-9583124-1-9.

McFARLAND, J.H., HATTON, R.M. and FOLEY, D.J. 1948. Garden bulbs in color. J. Horace McFarland Company, Harrisburg.

MACHOCHO, A., CHHABRA, S.C., VILADOMAT, F., CODINA, C. and BASTIDA, J. 1998. Alkaloids from *Crinum stuhlmannii*. *Planta Medica* 64: 679-680.

MAESATO, K., SHARADA, K., FUKUI, H., HARA, T. and SARMA, K.S. 1994. *In vitro* bulblet regeneration from bulbscale explants of *Lilium japonicum* Thunb. Effect of plant growth regulators and culture environment. *Journal of Horticultural Science* 69(2): 289-297.

MAMMEM, M. and CLOETE, E. 1996. Bridging the gap between traditional and "modern" medicine. *Veld & Flora* 82(1): 2.

MANSKE, R.H.F. 1975. The alkaloids: Their chemistry and physiology. Volume 15. Academic Press, New York.

MANTELL, S.H. and SMITH, H. 1983. Cultural factors that influence secondary metabolite accumulations in plant cell and tissue cultures. In: Mantell, S.H. and Smith, H. (Eds). Plant biotechnology. Cambridge University Press, Cambridge, pp. 75-108. ISBN 0-521-24550-8.

MARINANGELI, P. and CURVETTO, N. 1997. Increased sucrose and salt concentrations in the culture medium improve growth of micropropagated *Lilium* bulblets. *Biocell* 21: 161-164.

MARTIN, S.F. 1987. The Amaryllidaceae alkaloids. In: Brossi, A. (Ed). The alkaloids: Chemistry and pharmacology. Volume 30. Academic Press, San Diego, pp. 251-376.

MATHEW, B. and SWINDELLS, P. 1995. The complete book of bulbs, corms, tubers and rhizomes. RD Press, Surrey Hills (Australia). ISBN 0-86438-779-2.

MAVI, S. 1994. Medicinal plants and their uses in Zimbabwe. In: Huntley, B.J. (Ed). Botanical diversity in southern Africa. Strelitzia 1. National Botanical Institute, Pretoria, pp. 169-174. ISBN 1-87490-725-0.

MERKLE, S.A., PARROTT, W.A. and WILLIAMS, E.G. 1990. Applications of somatic embryogenesis and embryo cloning. In: Bhojwani, S.S. (Ed). Plant tissue culture: Applications and limitations. Elsevier, Amsterdam, pp. 54-66. ISBN 0-444-88883-7.

MII, M., MORI, T. and IWASE, N. 1974. Organ formation from the excised bulb scales of *Hippeastrum hybridum in vitro*. *Journal of Horticultural Science* 49: 241-244.

MISAWA, M. and NAKANISHI, T.M. 1988. Antitumour compounds: Production by cell cultures. In: Bajaj, Y.P.S. (Ed). Biotechnology in agriculture and forestry. Volume 4. Medicinal and aromatic plants. I. Springer-Verlag, Berlin, pp. 191-208. ISBN 3-540-18414-7.

MOCHTAK, E. 1989. Bulblet formation from the explants of *Nerine bowdenii* W.Watts. *Acta Horticulturae* 251: 199-204.

MOHAMED, Y.Y. 1999. *In vitro* propagation of grand crinum lily (*Crinum asiaticum* L.) from inflorescence. *Arab Universities Journal of Agricultural Sciences* 7(2): 521-529.

MORAES-CERDEIRA, R., BURANDT, C.L., BASTOS, J.K., NANAYAKKARA, N.P.D., MIKELL, J., THURN, J. and McCHESNEY, J.D. 1997. Evaluation of four *Narcissus* cultivars as potential sources of galanthamine production. *Planta Medica* 63: 472-474.

MUJIB, A., BANDOPADHYAY, S., JANA, B.K. and GHOSH, P.D. 1996. Growth regulators involvement and somatic embryogenesis in *Crinum asiaticum*. *Indian Journal of Plant Physiology* 1(2): 84-87.

MULLIN, M. 1970. Tissue culture of some monocotyledonous plants. *Australian Journal of Biological Science* 23: 473-477.

MURASHIGE, T. and SKOOG, F. 1962. A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiologia Plantarum* 15: 473-479.

MURASHIGE, T. 1974. Plant propagation through tissue cultures. *Annual Review of Plant Physiology* 25: 134-166.

MURAV'-EVA, D.A. and POPOVA, O.I. 1989. Polysaccharides of *Cochicum speciosum* and *Crinum amabile*. *Khimiko Farmatsevticheskii Zhurnal* 23(5): 619-620.

NAGASAWA, A. and FINER, J.J. 1988. Development of morphogenic suspension cultures of garlic (*Allium sativum* L.). *Plant Cell, Tissue and Organ Culture* 15: 183-187.

NAIR, A.J., SUDHAKARAN, P.R., RAO, J.M. and RAMAKRISHNA, S.V. 1992. Berberine synthesis by callus and cell suspension cultures of *Coscinium fenestratum*. *Plant Cell, Tissue and Organ Culture* 29: 7-10.

NAIR, J.J., CAMPBELL, W.E., GAMMON, D.W., ALBRECHT, C.F., VILADOMAT, F., CODINA, C. and BASTIDA, J. 1998. Alkaloids from *Crinum delagoense*. *Phytochemistry* 49(8): 2539-2543.

NAIR, J.J., MACHOCHO, A.K., CAMPBELL, W.E., BRUN, R., VILADOMAT, F., CODINA, C. and BASTIDA, J. 2000. Alkaloids from *Crinum macowanii*. *Phytochemistry* 54: 945-950.

NAKANO, M., NIIMI, Y., KOBAYASHI, D. and WATANABE, A. 1999. Adventitious shoot regeneration and micropropagation of hybrid tuberous begonia (*Begonia* x *tuberhydria* Voss). *Scientia Horticulturae* 79: 245-251.

NEWTON, D.J. and BODASING, A. 1994. TRAFFIC. Wildlife trade monitoring and the South African plant trade. In: Huntley, B.J. (Ed). Botanical diversity in southern Africa. Strelitzia 1. National Botanical Institute, Pretoria, pp. 319-328. ISBN 1-87490-725-0.

NG, S.Y.C. 1988. *In vitro* tuberization in white yam (*Dioscorea rotundata* Poir). *Plant Cell, Tissue and Organ Culture* 14: 121-128.

NGUGI, G.W., JÄGER, A.K. and VAN STADEN, J. 1998. *In vitro* propagation of *Drimia robusta* Bak. *South African Journal of Botany* 64(4): 266-268.

NICHOLS, G. 1984. Some exciting plants from northern Zululand and Tongaland. *Veld & Flora* 70(3): 79-80.

NICKELL, L.G. 1980. Products. In: Staba, E.J. (Ed). Plant tissue culture as a source of biochemicals. CRC Press, Boca Raton, pp. 235 - 269. ISBN 0-8493-5557-5.

NIIMI, Y., NAKANO, M. and SAITO, S. 1997. Production of commercial *Lilium rubellum* Baker bulbs: Effects of volume renewal of liquid medium on *in vitro* growth, bulb rot infection during cold treatment, and post-*in-vitro* growth of bulblets. *Journal of the Japanese Society for Horticultural Science* 66(1): 113-119.

OHSHIKA, K. and IKEDA, H. 1994. Isolation and preservation of the living embryo sac of *Crinum asiaticum* L. var. *Japonicum* Baker. *Journal of Plant Research* 107: 17-21.

OKPO, S.O. and ADEYEMI, O.O. 1998. Effects of *Crinum glaucum* aqueous extract on intestinal smooth-muscle activity. *Phytotherapy Research* 12: 413-416.

OKUBO, H. 1993. *Hippeastrum* (Amaryllis). In: De Hertogh, A.A. and Le Nard, M. (Eds). 1993. The physiology of flower bulbs. A comprehensive treatise on the physiology and utilization of ornamental flowering bulbous and tuberous plants. Elsevier Science Publishers, Amsterdam, The Netherlands, pp. 321-334. ISBN 0-444-87498-4.

OKUBO, H., HUANG, C.W. and UEMOTO, S. 1990. Role of outer scale in twin-scale propagation of *Hippeastrum hybridum* and comparison of bulblet formation from single- and twin-scales. *Acta Horticulturae* 266: 59-65.

OLIVER, I.B. 1990. *Crinum bulbispermum* - the flower of the Orange Free State. *Veld & Flora* 76(2): 57.

OLIVIER, W. 1983. Rare Amaryllidaceae from Southern Africa. *Veld & Flora* 69(2): 162-165.

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

PARR, A.J., ROBINS, R.J. and RHODES, M.J.C. 1986. Product release from plant cells grown in culture. In: Morris, P., Scragg, A.H., Stafford, A. and Fowler, M.W. (Eds). Secondary metabolism in plant cell cultures. Cambridge University Press, Cambridge. ISBN 0 521 32889 6.

PECK, D.E. and CUMMING, B.G. 1986. Beneficial effects of activated charcoal on bulblet production in tissue cultures of *Muscari armeniacum*. *Plant Cell, Tissue and Organ Culture* 6: 9-14.

PHAM, L.H., DÖPKE, W., WAGNER, J. and MÜGGE, C. 1998. Alkaloids from *Crinum amabile*. *Phytochemistry* 48(2): 371-376.

PHILLIPSON, J.D. 1990. Possibilities of finding new products from plant cell cultures. In: Nijkamp, H.J.J., van der Plas, L.H.W. and van Aartrijk, J. (Eds). Current plant science and biotechnology in agriculture. Progress in plant cellular and molecular biology. Kluwer Academic Publishers, Dordrecht, The Netherlands, pp. 592-600. ISBN 0-7923-0873-5.

PIERIK, R.L.M. 1987. *In vitro culture of higher plants*. Nijhoff, Dordrecht. ISBN -90-247-3531-9.

PIERIK, R.L.M. 1991. Micropropagation of ornamental plants. *Acta Horticulturae* 289: 45-53.

PIERIK, R.L.M. and IPPEL, B.J. 1977. Plantlet formation from excised bulb scale segments of *Nerine*. *Acta Horticulturae* 78: 197-202.

PIERIK, R.L.M. and POST, A.J.M. 1975. Rapid vegetative propagation of *Hyacinthus orientalis* L. *in vitro*. *Scientia Horticulturae* 3: 293-297.

PIERIK, R.L.M. and RUIBING, M.A. 1973. Regeneration of bulblets on bulb scale segments of hyacinth *in vitro*. *Netherlands Journal of Agricultural Science* 21: 129-138.

PIERIK, R.L.M. and STEEGMANS, H.H.M. 1986. Adventitious plantlet regeneration from floral stem explants of *Nerine bowdenii* W.Watts. *Netherlands Journal of Agricultural Science* 34: 217-223.

PIERIK, R.L.M., SPRENKELS, P.A. and VAN BRAGT, J. 1983. Rapid vegetative propagation of *Eucharis grandilfora in vitro*. *Acta Horticulturae* 147: 179-186.

PIERIK, R.L.M., BLOKKER, J.S., DEKKER, M.W.C., DE DOES, H., KUIP, A.C., VAN DER MADE, T.A., MENTEN, Y.M.J., DE VETTEN, N.C.M.H. 1990. Micropropagation of *Hippeastrum* hybrids. In: De Jong, J. (Ed). Integration of *in vitro* techniques in ornamental plant breeding. Proceedings, Symposium, 10-14 November 1990. EUCARPIA, Wageningen, pp. 21-27.

PRAKASH, J. and PIERIK, R.L.M. 1993. Plant biotechnology. Commercial prospects and problems. Intercept Ltd., Andover. ISBN 0-946707-98-7.

PRANCE, G.T., CHADWICK, D.J. and MARSH, J. 1994. Ethnobotany and the search for new drugs. John Wiley & Sons, Chichester. ISBN 0-471-95024-6.

PREIL, W., FLOREK, P., WIX, U. and BECK, A. 1988. Towards mass propagation by use of bioreactors. *Acta Horticulturae* 226: 99-106.

PREIL, W. 1991. Application of bioreactors in plant micropropagation. In: Debergh, P.C. and Zimmerman, R.H. (Eds). Micropropagation: Technology and Application. Kluwer Academic Publishers, pp. 425-455. ISBN 0-7923-0818-2.

PUJOL, J. 1990. Natur Africa. The herbalist handbook. African flora, medicinal plants. Jean Pujol Natural Healers Foundation, Durban. ISBN 0-620-15148-X.

RAFFAUF, R.F. 1996. Plant alkaloids. A guide to their discovery and distribution. Haworth, New York. ISBN 1-560-22860-1.

RAMADAN, M.A., KAMEL, M.S., OHTANI, K., KASAI, R. and YAMASAKI, K. 2000. Minor phenolics from *Crinum bulbispermum* bulbs. *Phytochemistry* 54: 891-896.

RAUWALD, H.W., KOBER, M. and DIETRICH, A. 1991. Are galanthamine and lycorine chemotaxonomical markers in the genus *Gethyllis*. *Planta Medica* 57: A107-A108.

RAZAFIMBELO, J., ANDRIANTSIFERANA, M., BAUDOUIN, G. and TILLEQUIN, F. 1996. Alkaloids from *Crinum firmifolium* var. *Hygrophilum*. *Phytochemistry* 41(1): 323-326.

READ, M. 1989. Over exploitation of wild bulbs by the horticultural trade. *Herbertia* 45: 6-12.

REES, A.R. 1992. Ornamental bulbs, corms and tubers. Commonwealth Agricultural Bureaux, International, Wallingford. ISBN 0-85198-656-0.

REMOTTI, P.C. 1995. Primary and secondary embryogenesis from cell suspension cultures of *Gladiolus*. *Plant Science* 107: 205-214.

REMOTTI, P.C. and LÖFFLER, H.J.M. 1995. Callus induction and plant regeneration from gladiolus. *Plant Cell, Tissue and Organ Culture* 42: 171-178.

RICE, R.D., ALDERSON, P.G. and WRIGHT, N.A. 1983. Induction of bulbing of tulip shoots *in vitro*. *Scientia Horticulturae* 20: 377-390.

ROBBERTSE, P.J., SMALL, J.G.C. and VON TEICHMAN, I. 1983. The ovule and aspects of germination in *Crinum bulbispermum* and *Crinum macowanii*. *South African Journal of Botany* 2(1): 57-62.

ROBERTS, M.F. 1988. Isoquinolines (*Papaver* alkaloids). In: Constabel, F. and Vasil, I.K. (Eds). Cell culture and somatic cell genetics of plants. Volume 5. Phytochemicals in plant cell cultures. Academic Press, Inc., San Diego, pp. 315-334. ISBN 0-12-715005-6.

ROBERTS, M. 1990. Indigenous healing plants. Southern Book Publishers, Halfway House, Johannesburg. ISBN 1-86812-317-0.

RUDGE, K. and MORRIS, P. 1986. The effect of osmotic stress on growth and alkaloid accumulation in *Catharanthus roseus*. In: Morris, P., Scragg, A.H., Stafford, A. and Fowler, M.W. (Eds). Secondary metabolism in plant cell cultures. Cambridge University Press, Cambridge. ISBN 0 521 32889 6.

SCOTT, G. 1993. Medicinal and aromatic plants. Healthcare, economics and conservation in South Africa. *Veld & Flora* 79(3): 84-87.

SCOTT-SHAW, C.R. 1999. Rare and threatened plants of KwaZulu-Natal and neighbouring regions. KwaZulu-Natal Nature Conservation Service, Pietermaritzburg. ISBN 0-620-24688-X.

SEABROOK, J.E.A., CUMMING, B.G. and DIONNE, L.A. 1976. The *in vitro* induction of adventitious shoot and root apices on *Narcissus* (daffodil and narcissus) cultivar tissue. *Canadian Journal of Botany* 54: 814-819.

SEABROOK, J.E.A. and CUMMING, B.G. 1977. The *in vitro* propagation of amaryllis (*Hippeastrum spp.* hybrids). *In Vitro* 13(12): 831-836.

SEABROOK, J.E.A. and CUMMING, B.G. 1982. *In vitro* morphogenesis and growth of *Narcissus* in response to temperature. *Scientia Horticulturae* 16: 185-190.

SELLÉS, M., BASTIDA, J., VILADOMAT, F. and CODINA, C. 1997[a]. Quantitative evaluation of galanthamine and related alkaloids in wild plants and tissue culture of *Narcissus confusus* by high performance liquid chromatography. *Analusis* 25: 156-158.

SELLÉS, M., BERGOÑÓN, S., VILADOMAT, F., BASTIDA, J. and CODINA, C. 1997[b]. Effect of sucrose on growth and galanthamine production in shoot-clump cultures of *Narcissus confusus* in liquid-shake culture. *Plant Cell, Tissue and Organ Culture* 49: 129-136.

SELLÉS, M., VILADOMAT, F., BASTIDA, J. and CODINA, C. 1999. Callus induction, somatic embryogenesis and organogenesis in *Narcissus confusus*: correlation between the state of differentiation and the content of galanthamine and related alkaloids. *Plant Cell Reports* 18: 646-651.

SHERRIFFS, P. 1995. Former foes set out to save muti plants. *The Natal Witness*, August 22.

SHIELDS, R., ROBINSON, S.J. and ANSLOW, P.A. 1984. Use of fungicides in plant tissue culture. *Plant Cell Reports* 3: 33-36.

SIMMONDS, J.A. and WERRY, T. 1987. Liquid-shake culture for improved micropropagation of *Begonia* x *hiemalis*. *HortScience* 22(1): 122-124.

SINGH, R.P. and PANT, N.C. 1980. Investigation on the antifeedant property of subfamily Amaryllidoideae (Amaryllidaceae) against desert locust, *Schistocerca gregaria* Forsk. *Indian Journal of Entomology* 42(3): 465-468.

SINGH, Y.P., KUMAR, Vijar and KUMAR, V. 1996. Repellent properties of some extracts against *Statherotis (Argyroploce) luecaspis* Meyr. *Recent Horticulture* 3(1): 132-133.

SLABBERT, R. 1989. Bewaar *Crinum* - 'n belangrike blombolplant in die RSA. *Roodeplaat Bulletin* 23.

SLABBERT, M.M. 1993. Pot plants: Rapid multiplication of bulb flower pot plants *in vitro*: *Crinum macowanii*. 1987 - 1991. In: Republic of South Africa - Agricultural Research 1993, Department of Agriculture. ISBN 0-621-14973-X.

SLABBERT, M.M., DE BRUYN, M.H., FERREIRA, D.I. and PRETORIUS, J. 1993. Regeneration of bulblets from twin-scales of *Crinum macowanii in vitro*. *Plant Cell, Tissue and Organ Culture* 33: 133-141.

SLABBERT, M.M., DE BRUYN, M.H., FERREIRA, D.I. and PRETORIUS, J. 1995. Adventitious *in vitro* plantlet formation from immature floral stems of *Crinum macowanii*. *Plant Cell, Tissue and Organ Culture* 43: 51-57.

SMITH, D.L. and KRIKORIAN, A.D. 1991. Growth and maintenance of an embryogenic cell culture of Daylily (*Hemerocallis*) on hormone-free medium. *Annals of Botany* 67: 443-449.

SMITS, P.C.H., PIERIK, R.L.M. and SCHEL, J.H.N. 1989. An anatomical study of the *in vitro* bulblet regeneration from flowering stem explants of *Hippeastrum* cv. Liberty. *Acta Botanica Neerlandica* 38: 348.

SNIJMAN, D.A. and LINDER, H.P. 1996. Phylogenetic relationships, seed characters, and dispersal system evolution in Amaryllideae (Amaryllidaceae). *Annals of the Missouri Botanical Garden* 83: 362-386.

SPOERKE, D.G. and SMOLINSKE, S.C. 1990. Toxicity of houseplants. CRC Press, Boca Raton, pp. 108-109. ISBN 0-8493-6655-0.

SQUIRES, W.M. and LANGTON, F.A. 1990. Potential and limitations of narcissus micropropagation: An experimental evaluation. *Acta Horticulturae* 266: 67-76.

SQUIRES, W.M., LANGTON, F.A. and FENLON, J.S. 1991. Factors influencing the transplantation success of micropropagated *Narcissus* bulbils. *Journal of Horticultural Science* 66(6): 661-671.

SRAMEK, J.J., FRACKIEWICZ, E.J. and CUTLER, N.R. 2000. Review of the acetylcholinesterase inhibitor galanthamine. *Expert Opinion on Investigational Drugs* 9: 2393-2402.

STABA, E.J. 1980. Plant tissue culture as a source of biochemicals. CRC Press, Boca Raton. ISBN 0-8493-5557-5.

STANILOVA, M.I., ILCHEVA, V.P. and ZAGORSKA, N.A. 1994. Morphogenetic potential and *in vitro* micropropagation of endangered plant species *Leucojum aestivum* L. and *Lilium rhodopaeum* Delip. *Plant Cell Reports* 13: 451-453.

STEINITZ, B. and YAHEL, H. 1982. *In vitro* propagation of *Narcissus tazetta*. *HortScience* 17(3): 333-334.

STEINITZ, B. and LILIEN-KIPNIS, H. 1989. Control of precocious *Gladiolus* corm and cormel formation in tissue culture. *Journal of Plant Physiology* 135: 495-500.

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.

STIRTON, C.H. 1980. Aspects of research on South African petaloid monocotyledons of horticultural importance. In: Brickell, C.D., Cutler, D.F. and Gregory, M. (Eds). Petaloid monocotyledons: Horticultural and botanical research. Papers presented at a symposium: Research into monocotyledons of horticultural importance; organized by the Linnanean Society of London and the Royal Horticultural Society, London 3-5 April 1979. Academic Press, London, pp. 191-197. ISBN 0-12-133950-5.

STÖCKIGT, J., OBITZ, P., FALKENHAGEN, R., LUTTERBACH, R. AND ENDREβ, S. 1995. Natural products and enzymes from plant cell cultures. *Plant Cell, Tissue and Organ Culture* 43: 97-109.

STREAK, D. 1995. Back to our roots. *Fair Lady*, 20 September, 1995, pp.109-113, 163-165.

SUHADOLNIK, R.J. 1964. Amaryllidaceae alkaloid formation by floral primordial tissue and callous tissue. *Lloydia* 27(4): 315-321.

TABATA, M. 1977. Recent advances in the production of medicinal substances by plant cell cultures. In: Barz, W., Reinhard, E. and Zenk, M.H. (Eds). Plant tissue culture and its bio-technological application. Springer-Verlag, Berlin. ISBN 3-540-08227-1.

TAEB, A.G. and ALDERSON, P.G. 1990. Effect of low temperature and sucrose on bulb development and on the carbohydrate status of bulbing shoots of tulip *in vitro*. *Journal of Horticultural Science* 65(2): 193-197.

TAKAHASHI, S., MATSUBARA, K., YAMAGATA, H. and MORIMOTO, T. 1992[b]. Micropropagation of virus free bulblets of *Lilium longiflorum* by tank culture. 1. Development of a liquid culture method and large scale propagation. *Acta Horticulturae* 319: 83-88.

TAKAHASHI, S., KATOH, A. and MORIMOTO, T. 1992. Micropropagation of virus free bulblets of *Lilium longiflorum* by tank culture. 2. Cultivation characteristic of propagated bulblet. *Acta Horticulturae* 319: 89-94.

TAKATORI, F.H., MURASHIGE, T. and STILLMAN, J.I. 1968. Vegetative propagation of Asparagus through tissue culture. *HortScience* 3(1): 20-22.

TAKAYAMA, S. 1991. Mass propagation of plants through shake and bioreactor culture techniques. In: Bajaj, W.P.S. (Ed). Biotechnology in agriculture and forestry. Hightech and micropropagation 1. Vol. 17. Springer-Verlag, pp. 1-46.

TAKAYAMA, S. and MISAWA, M. 1979. Differentiation in *Lilium* bulbscales grown *in vitro*. Effect of various cultural conditions. *Physiologia Plantarum* 46: 184-190.

TAKAYAMA, S. and MISAWA, M. 1980. Differentiation in *Lilium* bulbscales grown *in vitro*. Effects of activated charcoal, physiological age of bulbs and sucrose concentration on differentiation and scale leaf formation *in vitro*. *Physiologia Plantarum* 48: 121-125.

TAKAYAMA, S. and MISAWA, M. 1983[a]. A scheme for mass propagation of *Lilium in vitro*. *Scientia Horticulturae* 18: 353-362.

TAKAYAMA, S. and MISAWA, M. 1983[b]. The mass propagation of *Lilium in vitro* by stimulation of multiple adventitious bulb-scale formation and by shake culture. *Canadian Journal of Botany* 61: 224-228.

TAKAYAMA, S., AMO, T. and FUKANO, M. 1991[a]. Rapid clonal propagation of *Hyacinthus orientalis* bulbs by shake culture. *Scientia Horticulturae* 45: 315-321.

TAKAYAMA, S., SWEDLUND, B. and MIWA, Y. 1991[b]. Automated propagation of microbulbs of lilies. In: Vasil, I.K. (Ed). Cell culture and somatic cell genetics of plants. Vol. 8. Scale-up and automation in plant propagation. Academic Press, New York, pp. 111-131.

TAYLOR, J.L.S. and VAN STADEN, J. 1997. *In vitro* propagation of *Velthemia bracteata* and *V. bracteata* "Lemon Flame". *South African Journal of Botany* 63(3): 159-161.

TERZI, M. and LOSCHIAVO, F. 1990. Somatic embryogenesis. In: Bhojwani, S.S. (Ed). Plant tissue culture: Applications and limitations. Elsevier, Amsterdam, pp. 54-66. ISBN 0-444-88883-7.

THOMAS, M. Indigenous bulbous plants and their cultivation. In: Growing wild flowers. The Botanical Society of South Africa.

THOMPSON, P. 1989. Creative propagation: A grower's guide. Christopher Helm, London. ISBN 0-7470-3213-0.

THURSTON, K.C., SPENCER, S.J. and ARDITTI, J. 1979. Phytotoxicity of fungicides and bactericides in orchid culture media. *American Journal of Botany* 66(7): 825-835.

TICHA, I., CAP, F., PACOVSKA, D., HOFMAN, P., HAISEL, D., CAPKOVA, V. and SCHAFER, C. 1998. Culture on sugar medium enhances photosynthetic capacity and high light resistance of plantlets grown *in vitro*. *Physiologia Plantarum* 102(2): 155-162.

ULRICH, M.R., DAVIES, F.T., KOH, Y.C., DURAY, S.A. and EGILLA, J.N. 1999. Micropropagation of *Crinum* 'Ellen Bosanquet' by tri-scales. *Scientia Horticulturae* 82: 95-102.

USHER, G. 1974. A dictionary of plants used by Man. Constable, London. ISBN 0-09-457920-2.

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: 297-302.

VAN AARTRIJK, J. and VAN DER LINDE, P.C.G. 1986. *In vitro* propagation of flower bulb crops. In: Zimmerman, R.H., Hammerschlag, F.A. and Lawson, R.H. (Eds). Tissue culture as a plant production system for horticultural crops. Conference on tissue culture as a plant production system for horticultural crops, Beltsville, Md. October 20-23, 1985. Martinus Nijhoff Publishers, Dordrecht, pp. 317-331. ISBN 90-247-3378-2.

VAN DER LINDE, P.C.G. 1992. Tissue culture of flower-bulb crops. Theory and practice. *Acta Horticulturae* 325: 419-427.

VAN DER LINDE, P.C.G., HOL, G.M.G.M., BLOM-BARNHOORN, G.J., VAN AARTRIJK, J. and DE KLERK, G.J. 1988. *In vitro* propagation of *Iris hollandica* Tub. cv. Prof. Blaauw. I. Regeneration on bulb-scale explants. *Acta Horticulturae* 226: 121-128.

VAN DER POST, L. and TAYLOR, J. 1984. Testament to the Bushmen. Viking, Harmondsworth. ISBN 0-670-80065-1.

VELTEN, R., ERDELEN, C., GEHLING, M., GOHRT, A., GONDOL, D., LENZ, J., LOCKHOFF, O., WACHENDORFF, U. and WENDISCH, D. 1998. Cripowellin-A and Cripowellin-B, a novel type of Amaryllidaceae alkaloid from *Crinum powellii*. *Tetrahedron Letters* 39: 1737-1740.

VERDOORN, I.C. 1973. The genus *Crinum* in Southern Africa. *Bothalia* 11: 27-52.

VERPOORTE, R. 1998. Metabolic engineering as a strategy to improve production of secondary metabolites in plants or plant cell cultures. *Acta Horticulturae* 457: 403-407.

VIDOR, M.A. 1997. Aseptic techniques for cleaning bulbs of several ornamental species cultured *in vitro*. *Agropecuária Catarinense* 10(2): 49-54.

VILADOMAT, F., BASTIDA, J., CODINA, C., CAMPBELL, W.E. and MATHEE, S. 1995. Alkaloids from *Boophane flava*. *Phytochemistry* 40: 307-311.

VILADOMAT, F., BASTIDA, J., CODINA, C., NAIR, J. and CAMPBELL, W.E. 1997. Alkaloids of the South African Amaryllidaceae. *Recent Research Developments in Phytochemistry* 1: 131-171.

VISHNEVETSKY, J., ZAMSKI, E. and ZIV, M. 1997. Bulb growth and carbohydrate metabolism in *Nerine sarniensis* cultured *in vitro*. *Acta Horticulturae* 447: 213-214.

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

VRIJSEN, R., VANDEN BERGHE, D.A., VLIETINCK, A.J. and BOEYE, A. 1986. Lycorine: a eukaryotic termination inhibitor? *The Journal of Biological Chemistry* 261: 505-507.

WAGNER, H., HIKINO, H. and FARNSWORTH, N.R. 1988. Economic and medicinal plant research. Volume 2. Academic Press, London. ISBN 0-12-7300-62-7.

WALLER, G.R. and NOWACKI, E.K. 1978. Alkaloid biology and metabolism in plants. Plenum Press, New York. ISBN 0-306-30981-5.

WANG, P.J. and HUANG, L.C. 1976. Beneficial effects of activated charcoal on plant tissue and organ cultures. *In Vitro* 12(3): 260-262.

WANG, L., BAO, X-M., LIU, Y-H. and HAO, S. 1994. Origin of direct somatic embryos from cultured inflorescence axis segments of *Freesia refracta*. *International Journal of Plant Science* 155(6): 672-676.

WATAD, A.A., KOCHBA, M., NISSIM, A. and GABA, V. 1995. Improvement of *Aconitum napellus* micropropagation by liquid culture on floating membrane rafts. *Plant Cell Reports* 14: 345-348.

WATAD, A.A., AHRONI, A., ZUKER, A., SHEJTMAN, H., NISSIM, A. and VAINSTEIN, A. 1996. Adventitious shoot formation from carnation stem segments: A comparison of different culture procedures. *Scientia Horticulturae* 65: 313-320.

WATAD, A.A., ALPER, Y., STAV, R. and LEVIN, R. 1999. Mechanization of micropropagation. In: Altman, A. *et al.* (Eds). Plant biotechnology and *in vitro* biology in the 21st century. Kluwer Academic Publishers, The Netherlands, pp. 663-666.

WATT, J.M. and BREYER-BRANDWIJK, M.G. 1962. The medicinal and poisonous plants of southern and eastern Africa: Being an account of their medicinal and other uses, chemical composition, pharmacological effects and toxicology in man and animals. E & S Livingstone Ltd., Edinburgh, pp. 30 - 32.

WAREING, P.F. and JENNINGS, A.M.V. 1980. The hormonal control of tuberisation in potato. In: Skoog, F. (Ed). Plant growth substances 1979. Springer-Verlag, Berlin.

WEATHERHEAD, M.A., BURDON, J. and HENSHAW, G.G. 1978. Some effects of activated charcoal as an additive to plant tissue culture media. *Zeitschrift für Pflanzenphysiologie Bd.* 89: 141-147.

WENIGER, B., ITALIANO, L., BECK, J.P., BASTIDA, J., BERGOÑÓN, S., CODINA, C., LOBSTEIN, A. and ANTON, R. 1995. Cytotoxic activity of Amaryllidaceae alkaloids. *Planta Medica* 61(1): 77-79.

WILDMAN, W.C. 1960. Alkaloids of the Amaryllidaceae. In: Manske, R.H.F. and Holmes, H.L. (Eds). The alkaloids: Chemistry and physiology. Volume VI. Academic Press, New York.

WILLIAMS, V.L. 1996. The Witwatersrand muti trade. *Veld & Flora* 82(1): 12-14.

WILLIAMS, G. 2000. A year of Batten's African blooms. *The Natal Witness*, Saturday, November 11.

WINK, M. 1987. Physiology of the accumulation of secondary metabolites with special reference to alkaloids. In: Constabel, F. and Vasil, I.K. (Eds). Cell culture and somatic cell genetics of plants. Vol. 4. Cell culture in phytochemistry. Academic Press, San Diego, pp. 17-42. ISBN 0-12-715004-8.

WITHERS, L.A. 1991. *In vitro* conservation. *Biological Journal of the Linnean Society* 43: 31-42.

YAHIA, A., KEVERS, C., GASPAR, T., CHENIEUX, J.C., RIDEAU, M. and CRECHE, J. 1998. Cytokinins and ethylene stimulate indole alkaloid accumulation in cell suspension cultures of *Catharanthus roseus* by two distinct mechanisms. *Plant Science* 133: 9-15.

YAMADA, Y. and HASHIMOTO, T. 1990. Possibilities for improving yields of secondary metabolites in plant cell cultures. In: Nijkamp, H.J.J., van der Plas, L.H.W. and van Aartrijk, J. (Eds). Progress in plant cellular and molecular biology. Kluwer Academic Publishers, Dordrecht, pp. 547-556. ISBN -7923-0873-5.

YAMAGISHI, M. 1998. Effects of culture temperature on the enlargement, sugar uptake, starch accumulation, and respiration of *in vitro* bulblets of *Lilium japonicum* Thunb. *Scientia Horticulturae* 73(4): 239-247.

YASUDA, H., SHITOH, T., YAMANO, T., ITOH, Y. and SHIMURA, S. 1986. Identification of neokestose produced from sucrose by an enzyme of *Penicillium oxalicum*. *Agricultural and Biological Chemistry* (Tokyo) 50(3): 777-778.

YEOMAN, M.M. 1986. Plant cell culture technology. Botanical monographs Volume 23. Blackwell Scientific Publications, Oxford. ISBN 0-632-01393-1.

ZEL, J., DEBELJAK, N., UCMAN, R. and RAVNIKAR, M. 1997. The effect of jasmonic acid, sucrose and darkness on Garlic (*Allium sativum* L. Cv. Ptujski-Jesenski) bulb formation *in vitro*. *In Vitro Cellular and Developmental Biology - Plant* 33: 231-235.

ZENK, M.H., RÜFFER, M., KUTCHAN, T.M. and GALNEDER, E. 1988. Biotechnological approaches to the production of isoquinoline alkaloids. In: Bock, G. and Marsh, J. (Eds). Applications of plant cell and tissue culture. John Wiley and Sons, Chichester. ISBN 0-471-91886-5.

ZHAO, J., ZHU, W-H. and HU, Q. 2001. Effects of light and plant growth regulators on the biosynthesis of vindoline and other indole alkaloids in *Catharanthus roseus* callus cultures. *Plant Growth Regulation* 33(1): 43-49.

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[a]. Morphogenesis of gladiolus buds in bioreactors - implication for scale-up propagation of geophytes. In: Nijkamp, H.J.J., van der Plas, L.H.W. and van Aartrijk, J. (Eds). Current plant science and biotechnology in agriculture. Progress in plant cellular and molecular biology. Kluwer Academic Publishers, Dordrecht, The Netherlands. ISBN 0-7923-0873-5.

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

ZIV, M. 1991. Morphogenic patterns of plants micropropagated in shaken flasks or large scale bioreactor cultures. *Israel Journal of Botany* 40: 145-153.

Ziv, M. 1992[a]. The use of growth retardants for the regulation and acclimatization of *in vitro* plants. In: Karssen, C.M., van Loon, L.C. and Vreugdenhil, D. (Eds). Progress in plant growth regulation. Kluwer Academic Publishers, Dordrecht, pp. 809-817.

ZIV, M. 1992[b]. Morphogenic control of plants micropropagated in bioreactor cultures and its possible impact on acclimatization. *Acta Horticulturae* 319: 119-124.

ZIV, M. 1995. The control of bioreactor environment for plant propagation in liquid culture. *Acta Horticulturae* 393: 25-38.

ZIV, M. 1997. The contribution of biotechnology to breeding, propagation and disease resistance in geophytes. *Acta Horticulturae* 430: 247-258.

ZIV, M. 1999. Organogenic plant regeneration in bioreactors. In: Altman, A. *et al.* (Eds). Plant biotechnology and *in vitro* biology in the 21st century. Kluwer Academic Publishers, The Netherlands, pp. 673-676.

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

ZIV, M., KAHANY, S. and LILIEN-KIPNIS, H. 1994. Scaled-up proliferation and regeneration of *Nerine* in liquid cultures. Part 1. The induction and maintenance of proliferating meristematic clusters by paclobutrazol in bioreactors. *Plant Cell, Tissue and Organ Culture* 39: 109-115.

ZIV, M., KAHANY, S. and LILIEN-KIPNIS, H. 1995. Somatic embryos and bulblet development from bioreactor regenerated meristematic clusters of *Nerine*. *Acta Horticulturae* 393: 203-212.

ZIV, M. and LILIEN-KIPNIS, H. 1997[a]. Bud cluster proliferation in bioreactor cultures of *Ornithogalum dubium*. *Acta Horticulturae* 430: 307-310.

ZIV, M. and LILIEN-KIPNIS, H. 1997[b]. The inflorescence stalk: A source of highly regenerative explants for micropropagation of geophytes. *Acta Horticulturae* 447: 107-111.

ZIV, M. and LILIEN-KIPNIS, H. 2000. Bud regeneration from inflorescence explants for rapid propagation of geophytes in vitro. *Plant Cell Reports* 19: 845-850.