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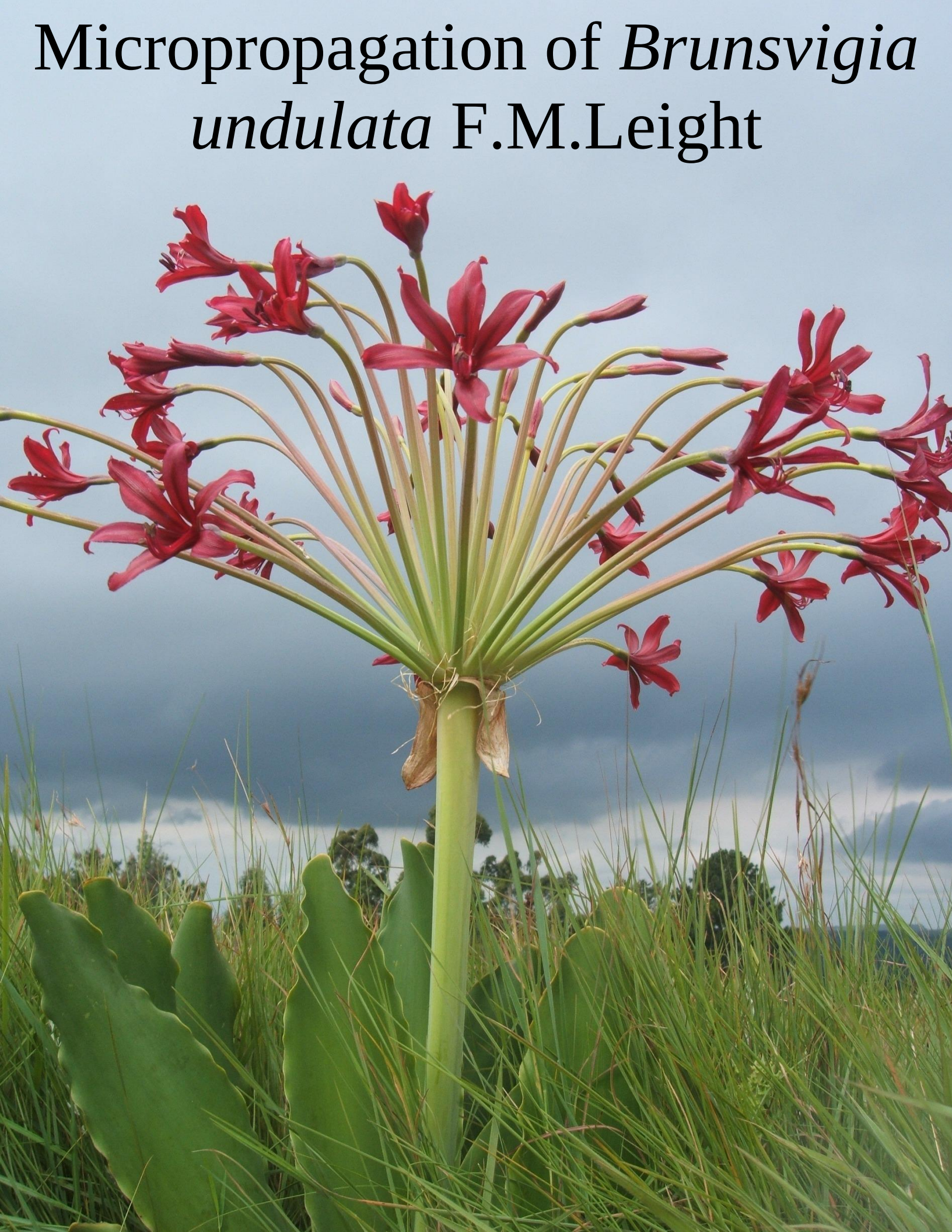
Micropropagation of *Brunsvigia undulata* F.M.Leight



Micropropagation of *Brunsvigia undulata* F.M. Leight

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STUDENT DECLARATION

Micropropagation of *Brunsvigia undulata* F.M. Leight

I Laura Jane Rice (student number) 203507134

declare that :

(i) The research reported in this dissertation, except where otherwise indicated, is the result of my own endeavours in the Research Centre for Plant Growth and Development, School of Biological and Conservation Sciences, University of KwaZulu-Natal Pietermaritzburg;

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Signed at Pietermaritzburg on the 27th day of January 2009.

_____

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DECLARATION BY SUPERVISORS

We hereby declare that we acted as Supervisors for this MSc student:

Student's Full Name: Laura Jane Rice

Student Number: 203507134

Thesis Title: Micropropagation of *Brunsvigia undulata* F.M. Leight

Regular consultation took place between the student and ourselves throughout the investigation. We advised the student to the best of our ability and approved the final document for submission to the Faculty of Science and Agriculture Higher Degrees Office for examination by the University appointed Examiners.

SUPERVISOR: _____ PROFESSOR J VAN STADEN

CO-SUPERVISOR: _____ DR JF FINNIE

Abstract

Many South African medicinal plants face the threat of over-collection for use in traditional medicines. Many bulbous plants suffer as the whole plant is removed from the wild so that the bulb may be used for medicine. Micropropagation is a technique which can be used as an alternative to conventional propagation methods. Micropropagation produces many plantlets in a relatively short period of time.

Different plant parts of *Brunsvigia undulata* F.M. Leight, a rare South African species of medicinal value, were used in an attempt to produce *in vitro* plantlets using micropropagation techniques. Although leaf and floral explants were successfully formed from seedling explants and twin-scales.

Seeds germinated quickly in culture. Seedlings which grew from seeds were cut into sections and used to initiate bulblets. Seedling explants formed bulblets, shoots and callus best when the explants included a meristematic region. Callus from seedling explants formed shoot clusters readily when placed on hormone-free MURASHIGE and SKOOG (1962) (MS) medium. Shoots from shoot clusters formed bulblets and rooted on medium supplemented with IBA. The greatest rooting response was achieved by bulblets on 1 mgl^{-1} IBA. The callus which was left after shoot clusters were separated was placed back onto hormone-free MS medium. Callus explants continued to form shoot clusters.

Twin-scales, cut from large parent bulbs, were cultured on 25 hormone treatments. Bulblets formed on twin-scales even in the absence of plant growth hormones. Bulblets formed by twin-scales were used to determine the effects of both medium constituents and environmental factors on bulblet multiplication. Bulblet multiplication was greatest when bulblets were split in half and cultured as half-bulblets. Optimal multiplication was achieved on hormone-free MS, with 4% sucrose, kept at high temperatures in the dark.

Bulblets were successfully initiated and multiplied from both seedlings and twin-scales. Bulblets which were produced via both protocols were acclimatized relatively easily. Both explant types could be used to mass propagate *Brunsvigia undulata*.

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List of abbreviations

ANOVA	analysis of variance
BA	N^6-benzyladenine
Benlate™	[methyl-(butylcarbamoyl)-2-benzimidazole-carbamate]; commercially available as Benlate
IBA	indole-3-butyric acid
MGT	mean germination time
MS	Murashige and Skoog medium (1962)
NAA	α-naphthalenacetic acid
rpm	rotations per minute
Tween 20™	brand name for *polyoxyethylene sorbitan monolaurate* mw 1227.54

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Conference contributions

RICE, L. J., FINNIE, J. F. & VAN STADEN, J. (2008). *In vitro* propagation of *Brunsvigia undulata* from seeds. Fourth World Congress on Medicinal and Aromatic Plants (WOCMAP). ICC, Cape Town.

RICE, L. J., FINNIE, J. F. & VAN STADEN, J. (2008). Tissue Culture of *Brunsvigia undulata*. Thirty-Fourth Annual Conference of the South African Association of Botanists (SAAB). University of Johannesburg, Drakensville.

Chapter 1

INTRODUCTION

Southern Africa is home to 10% of the worlds higher plants (VAN WYK & GERICKE, 2000). Thirty thousand flowering plants makes this region an incredibly rich centre of plant diversity. Many of these flowering plants grow from bulbs hidden beneath the ground. Southern Africa has more bulbs than any other region (FENNELL, 2002) and is often called the "Bulb Capital of the World" (FENNELL & VAN STADEN, 2004).

Added to this floral diversity, South Africa has a rich cultural diversity. The local people of South Africa have used indigenous plants to treat a variety of ailments. Many of the indigenous plant species have become part of the traditions and folklore of the indigenous people (FENNELL & VAN STADEN, 2004). Today traditional healers are still very much a part of daily life. Roots, bulbs, stems and leaves are prepared in a variety of ways and administered orally, as an enema or applied directly onto wounds.

Cultivation of South African medicinal plants is minimal. It was suggested as an alternative to collecting plants from the wild over fifty years ago (GERSTNER, 1938; cited by CUNNINGHAM, 1990). However, there is still no large scale cultivation of medicinal plants (FENNELL, 2002). Unfortunately with a decrease in natural areas and an increase in demand for traditional medicine, many medicinal plants are threatened by over collection. The demand for traditional medicine is increasingly putting strain on wild populations of medicinal plants (POOLEY, 1998). Trade in bulbs is centuries old (READ, 1989) and is unfortunately unlikely to cease. A means for the propagation including micropropagation of these plants will relieve the pressure on plants growing in the wild.

Plants belonging to the genus *Brunsvigia* Heist. are both beautiful and poisonous (WATT & BREYER-BRANDWIJK, 1962). The bulbs of these toxic plants are used by the Zulu, Xhosa and southern Sotho people for their medicinal properties. Bulbs are collected from

the wild to be sold in traditional herbal markets and are used by some 200 000 traditional medical practioners in South Africa (NIELSEN *et al*., 2004). The medicinal properties of *Brunsvigia* are derived from the alkaloids which they produce. These alkaloids have pharmacological effects (NIELSEN *et al*., 2004). Tissue culture may also provide an alternative method for the production of useful alkaloids *in vitro*.

1.1 DISTRIBUTION AND MORPHOLOGY

There are 17 *Brunsvigia* species which grow in various parts of southern Africa (SNIJMAN & ARCHER, 2003), 16 of which are found in South Africa (POOLEY, 1998). Four species namely, *Brunsvigia radulosa*, *Brunsvigia natalensis*, *Brunsvigia undulata* and *Brunsvigia grandiflora* are found in KwaZulu-Natal. These plants grow in grasslands (CROUCH *et al.*, 2002) throughout the country (Figure 1.5). Those growing in the winter rainfall areas such as the Western Cape and Namaqualand flower in autumn. The species which grow in KwaZulu-Natal flower in late summer after the summer rains (MANNING *et al.*, 2002). *Brunsvigia undulata* is found growing in the KwaZulu-Natal midlands and the lower Drakensberg (Figure 1.1).

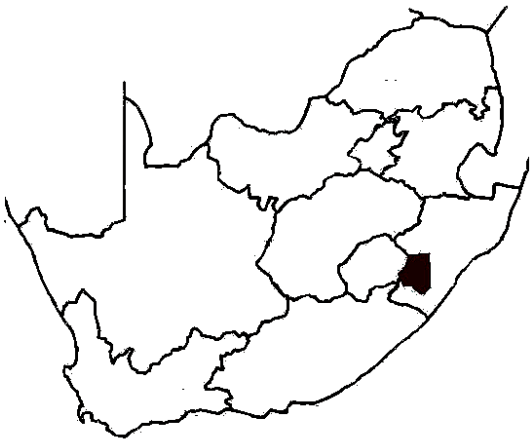


Figure 1.1: Shaded area shows the distribution of *Brunsvigia undulata* in South Africa.

Brunsvigia species bear flowers in an umbel which is characteristic of the Amaryllidaceae (Figure 1.2 A). The flowers vary from white to shades of pink and red (DU PLESSIS & DUNCAN, 1989). The number of flowers on an inflorescence varies

between few in certain species and many in others (DYER, 1976; OLIVIER, 1983). Up to 80 flowers may be borne in a single umbel. Flowers are on long stalks which together form a ball like structure, an umbel (Figure 1.2 A). The large inflorescence is supported by a thick, flattened, fleshy peduncle. The umbel dries during fruiting (Figure 1.2 B). Once the seed is ripe the fruiting head breaks off from the plant and rolls around in the wind distributing seeds (VAN WYK & GERICKE, 2000). The seeds are succulent, green and pea-shaped (OLIVIER, 1983). As with most members of the Amaryllidaceae the seeds of *Brunsvigia* are recalcitrant.

The peduncle protrudes from strap like leaves (Figure 1.2) which may be almost upright or flat against the ground (POOLEY, 1998). The leaves are also quite varied between species. Leaves are smooth in certain species and hairy in others (MANNING *et al.*, 2002). The leaf margins are often wavey (Figure 1.4) as in *Brunsvigia grandiflora* and *Brunsvigia undulata* (POOLEY, 1998).

The bulbs (Figure 1.4) of this genus vary in size depending on the species. *Brunsvigia comptonii* have small bulbs with a diameter of between 2.5 and 4 cm (MANNING *et al.*, 2002). At the other end of the spectrum *Brunsvigia josephinae* have huge bulbs which are 20 cm in diameter (DU PLESSIS & DUNCAN, 1989). The bulbs are subterranean in most species however some species have bulbs which are partly above ground (HUTCHINGS *et al*., 1996).

1.2 PLANT USES

1.2.1 Medicinal uses

Many of the Amaryllidaceae are used in southern Africa for their healing properties. Different parts of *Brunsvigia* plants are used by the Zulu, Xhosa and southern Sotho in their traditional medicine. *Brunsvigia* is also a part of traditional medicine in Swaziland and Lesotho (CROUCH *et al*., 2002).

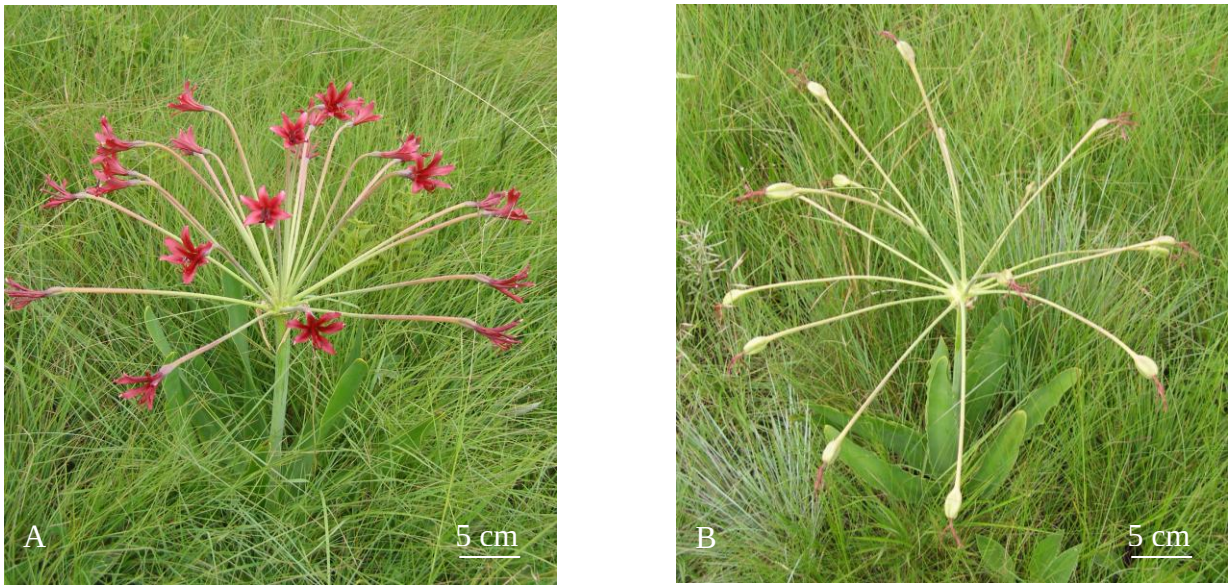


Figure 1.2: *Brunsvigia undulata* in flower (A) and while fruiting (B).

Figure 1.3: The ripe seeds (A) and the flower (B) of *Brunsvigia undulata*.

Figure 1.4: The bulb and leaves of *Brunsvigia undulata.*

Figure 1.5: Collecting *Brunsvigia undulata* from the grassland at Mount Gilboa.

HUTCHINGS *et al*. (1996) explain that the Zulu treat coughs and colds with bulb decoctions made by boiling the bulb in water. Bulbs are used to make enemas which are a treatment for renal and liver complaints. Enemas made from the bulb are also used to treat abdominal complaints (WATT & BREYER-BRANDWIJK, 1962; HUTCHINGS *et al*., 1996; CROUCH *et al*., 2002). Enemas are administered by the southern Sotho to relieve back pain (WATT & BREYER-BRANDWIJK, 1962). The scales of the bulb are used to sooth and heal wounds (POOLEY, 1998). The Xhosa use the outer scales of the bulbs to dress circumcision wounds as they promote very rapid healing (HUTCHINGS *et al*., 1996).

Bulbs are used as purgatives and bulb infusions as emetics (HUTCHINGS *et al*., 1996). Both the Swazi and the Zulu use the plant to straighten bones in children (POOLEY, 1998; CROUCH *et al*., 2002). Barren women are treated with a combination of *Brunsvigia radulosa* and other plants (CROUCH *et al*., 2002). POOLEY (1998) states that *Brunsvigia radulosa* is used to ease birth. The Sotho people use *Brunsvigia radulosa* during problematic child birth (CROUCH *et al*., 2002).

Interpretations of rock art suggest that *Brunsvigia* species were used by the San people to induce hallucinations and trance states (CROUCH *et al*., 2002). Hallucinations have been reported by individuals who have ingested parts of Amaryllidaceae bulbs. *Brunsvigia* is used to treat mental disorders such as schizophrenia or depression (CROUCH *et al*., 2002; NIELSEN *et al*., 2004).

CROUCH *et al.* (2002) reported that *Brunsvigia radulosa* has been used by traditional healers to treat cancer, and that extracts from this plant have antineoplastic activity. CAMPBELL *et al.* (2000) state that extracts from *Brunsvigia radulosa* had an inhibitory effect on lymphocytic leukaemia in mice.

The alkaloids extracted from *Brunsvigia* have an inhibitory effect on *Plasmodium falciparum* (CAMPBELL *et al*., 2000), the protozoan parasite which causes malaria in humans.

1.2.1.1 Alkaloids from *Brunsvigia* species.

The use of alkaloid-containing plants for medicines, potions and poisons can be traced back to the beginning of civilization (KUTCHAN, 1995). The medicinal properties of *Brunsvigia* are attributed to the alkaloids which are produced by the plant. Although there are many different alkaloids in each plant some of the more abundant ones have been isolated and identified. Many of the alkaloids extracted from *Brunsvigia* are common to other members of the Amaryllidaceae family.

One of the most significant alkaloids found in *Brunsvigia* species is lycorine (Figure 1.6 A). This cytotoxic compound is found throughout the genus (WATT & BREYER-BRANDWIJK, 1962; HUTCHINGS *et al.*, 1996; CROUCH *et al*., 2002) and is the most abundant alkaloid in the Amaryllidaceae family (NEUWINGER, 1996). NEUWINGER (1996) lists the numerous pharmacological effects of lycorine. This alkaloid has an emetic effect and hypotensive action in cats and dogs. It eliminated the fever induced by caffeine and peptone and lowered the temperature of fever induced by the typhoid vaccine. Lycorine has shown both antimalarial and antineoplastic activity. It inhibits cell growth and division (EVIDENTE *et al*., 1983) and reduces the viability of tumour cells *in vitro* (NEUWINGER, 1996).

Crinamine (Figure 1.6 D) has been isolated from *Brunsvigia orientalis* (VILADOMAT *et al*., 1996), *Brunsvigia radulosa* (CROUCH *et al*., 2002) and *Brunsvigia josephinae* (VILADOMAT *et al*., 1995b). Crinamine has antibacterial properties (VILADOMAT *et al*., 1996) and slight antimalarial activity (VILADOMAT *et al*., 1996; CROUCH *et al*., 2002). Crinamine has been isolated from seven psychotropic amaryllids. CROUCH *et al*. (2002) postulated that this alkaloid is responsible for the psychoactivity of *Brunsvigia radulosa*.

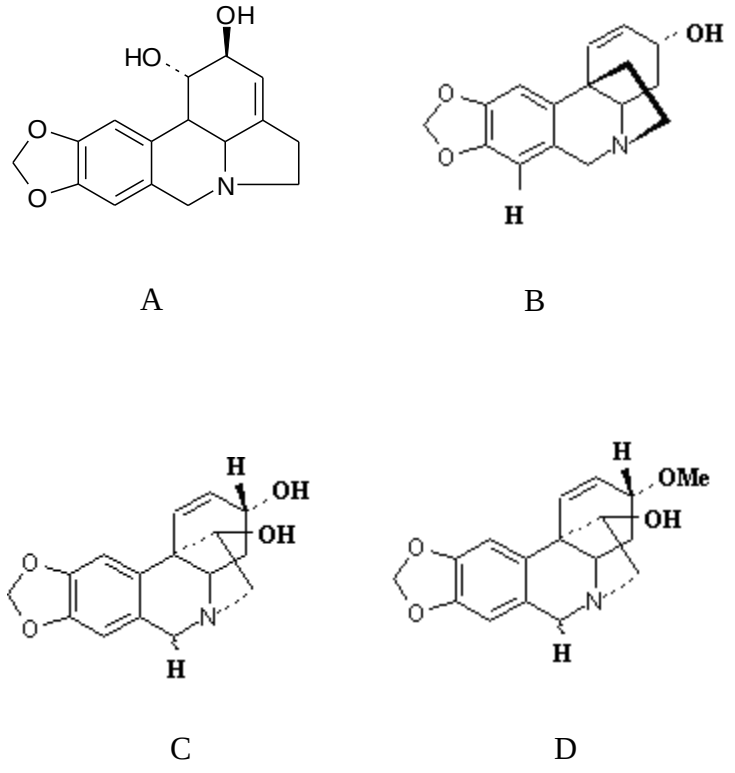


Figure 1.6: The chemical structures of lycorine (A), crinine (B), hamayne (C) and crinamine (D).

Another of the alkaloids isolated from *Brunsvigia orientalis*, undulatine is thought to be responsible for antineoplastic activity in *Amaryllis belladonna* (VILADOMAT *et al*., 1996). Buphanidrine has been isolated from *Brunsvigia josephinae* (VILADOMAT *et al*., 1995b). The literature does not mention the pharmacology of this alkaloid. However, NEUWINGER (1996) stated that it is not cytotoxic. Buphanine is an alkaloid also found in both *Brunsvigia* and *Boophone distichia* (DE SMET, 1996). HUTCHINGS *et al*. (1996) stated that this alkaloid is mydriatic, paralyses salivary secretion and the vagus terminations in the heart and produces death from respiratory failure. It has also been classed as cardioactive (HUTCHINGS & VAN STADEN, 1994).

CROUCH *et al*. (2002) isolated anhydrolycornium chloride from *Brunsvigia radulosa*. This alkaloid is known to occur in *Amaryllis belladonna* and has been shown to be a strong antineoplastic agent. When tested for its activity against lymphocytic leukaemia, anhyrolycornium chloride had an inhibitory effect on this leukaemia. Crinine (Figure 1.6 B) which has also been isolated from *Crinum* species is a cytotoxic alkaloid and has been

tested on human leukaemia cells (ABD EL HAFIZ *et al.*, 1991). Hamayne (Figure 1.6 C) has choline esterase inhibitory activity. This means that it can be used in the treatment of Alzheimer's disease (HOUGHTON *et al*., 2004). Both crinine and hamayne inhibit serotonin re-uptake in the rat brain. This means that both of these alkaloids could have potential in the treatment of depression (ELGORASHI *et al.*, 2006).

Many other alkaloids have been isolated from a number of *Brunsvigia* species. These include brunsvigine, sternbergine, belladine and josephinine (VILADOMAT *et al*., 1995a; VILADOMAT *et al*., 1996; CROUCH *et al*., 2002).

1.2.2 Traditional non-medicinal uses

Brunsvigia bulbs have been used to seal leaks in clay pots (POOLEY, 1998). "Witch doctors" use a method of divination to predict the future which is called 'throwing the bones'. These 'bones' are sometimes washed in a decoction made from the bulb which supposedly increases the accuracy of the method (WATT & BREYER-BRANDWIJK, 1962; CROUCH *et al*., 2002).

1.2.3 Horticultural uses

Many of the Amaryllidaceae are popular garden plants as they have very attractive flowers. The *Brunsvigia* genus is no exception. Their large and attractive blooms in several shades of red and pink make it a desirable addition to gardens and rockeries (DU PLESSIS & DUNCAN, 1989). Species such as *Brunsvigia undulata* have a fan arrangement of leaves (POOLEY, 1998) which adds to its aesthetic appeal. POOLEY (1998), suggests a number of the *Brunsvigia* species as good container plants and mentions that they are good feature plants.

Brunsvigia plants are sensitive and quite difficult to grow (PIENAAR, 1994). The seeds must be sown quickly after they ripen as they are viable for a short time only. Seedlings of the dwarf species can take up to four years to flower (DU PLESSIS & DUNCAN,

1989; PIENAAR, 1994) and they have long generation times (FENNELL & VAN STADEN, 2004). Larger species will mature for six or seven seasons before they flower (DU PLESSIS & DUNCAN, 1989). They are a genus for the patient gardener.

1.3 CONSERVATION

1.3.1 Conservation status of the Amaryllidaceae

According to the World Conservation Monitoring Centre, it has been estimated that 10% of the world's flora is currently under threat (FENNELL, 2002). KOOPOWITZ (1986) reported that 10-25% of higher plants are in danger of becoming extinct in the next 50 years. Twenty-two years later and few of these species have been 'saved'.

There are pressures on plants from industrial development, air and water pollution, farming, livestock grazing and the clearance of land for timber and crop production (MARSHAL, 1993; cited by NEWTON & BODASING, 1994). Population growth brings a requirement for land to be used for housing. It also increases the demand for traditional medicines which are being harvested in unsustainable ways (FENNELL & VAN STADEN, 2004). Plants used in traditional medicines are collected from the wild. Collection of medicinal plants from the wild is a source of income for people living in rural areas (AFOLAYAN & ADEBOLA, 2004).

In countries where bulbs are being collected from the wild to meet the demands by the horticultural world for novelty plants, there is concern for the preservation of these bulbs (FENNELL, 2002). The petaloid monocotyledons are "showy plants" and so are particularly vulnerable to over collection and exploitation" (STIRTON, 1980).

Of all Amaryllidaceae species in the world, 22% are found in South Africa and 66 are in various categories of endangerment (FENNELL, 2002). In KwaZulu-Natal the family is listed as specially protected (FENNELL, 2002).

Brunsvigia undulata populations have been put under pressure from the medicinal plant trade in South Africa, and in particular KwaZulu-Natal. So much so that the plant is rarely seen other than in grasslands which are part of privately owned land with restricted public access (Figure 1.5). Bulbs of closely related plant species such as *Boophone distichia* may be purchased at a medicinal plant market for a mere R5 each. *Brunsvigia undulata* bulbs were not seen at the markets throughout the course of this project. This is due to their scarceness in the wild.

Micropropagation, seed germination, regeneration from callus, embryo rescue, micrografting and cryopreservation are among the techniques used to conserve endangered plant species (CHANG *et al.*, 2000).

1.3.2 Conservation problems with the Amaryllidaceae

There are a number of characteristics of the family Amaryllidaceae, which when combined, make them difficult to conserve (KOOPOWITZ, 1986). Amaryllidaceae have long generation times (KOOPOWITZ, 1986) and can take up to seven seasons to flower for the first time (DU PLESSIS & DUNCAN, 1989). *Brunsvigia* species form offsets irregularly, if at all (DU PLESSIS & DUNCAN, 1989). This means that plant populations do not frequently regenerate themselves in the wild.

Recalcitrant seeds are fleshy and cannot be stored or cryogenically frozen (KOOPOWITZ, 1986). This excludes these seeds from being entered into genebanks and being preserved.

Members of this family are susceptible to disease (KOOPOWITZ, 1986) and also to pests (DODDS, 1991). Amaryllis lily borer feeds on all plant parts and can do huge damage to the foliage (Figure 1.7). This caterpillar also feeds on the seeds and eats away at the bulb until the plant dies.

A B

Figure 1.7: Amaryllis lily borer (arrows) on the dried umbel (A) and leaves (B) of *Brunsvigia undulata*.

1.4 PROPAGATION

1.4.1 Conventional methods of propagation

PIENAAR (1994) warns gardeners that *Brunsvigia* are harder to grow than many of the other Amaryllidaceae. The plants are easily propagated from seed but can take up to fourteen years to mature and flower (MANNING *et al.*, 2002). The roots do not do well when disturbed (DU PLESSIS & DUNCAN, 1989) and plants are known to flower erratically when taken out of their natural habitats (MANNING *et al*., 2002).

Seed must be planted deep in seed trays when it is ripe (DU PLESSIS & DUNCAN, 1989). They should be planted in a very sandy medium and need full sun (DU PLESSIS & DUNCAN, 1989; PIENAAR, 1994). Alternatively plants can be propagated by basal cuttage (DU PLESSIS & DUNCAN, 1989). The plants should be watered well during their growing season and not as much while they are dormant (PIENAAR, 1994).

1.4.2 Tissue Culture

Micropropagation protocols have been developed for geophytes from around the world. Those which have been concentrated on are ornamentals for which there is a demand in the horticultural market, others are medicinal plants or plants which are threatened in their natural habitat. Twin-scales have been the explant of choice in many of these protocols (Table 1.1).

Table 1.1: Plants where twin-scales were used as explants in tissue culture.

Family	Genus	References
Alliaceae	*Allium cepa*	DUNSTAN & SHORT, 1977
		HUSSEY & FALAVIGNA, 1980
Amaryllidaceae	*Amaryllis belladonna*	DE BRUYN *et al*., 1992
	Crinum macowanii	SLABBERT *et al*., 1993
	Crinum variabile	FENNELL *et al*., 2001
	Cyrtanthus clavatus	MORÁN *et al.*, 2003
	Cyrtanthus loddigesianus	ANGULO *et al*., 2003
	Cyrtanthus speciosus	ANGULO *et al*., 2003
	Cyrtanthus spiralis	MORÁN *et al.*, 2003
	Eucrosia stricklandii	COLQUE *et al*., 2002
	Gethyllis sp.	NIEDERWIESER *et al*., 2002
	Leucojum sp.	KROMER, 1985
	Lycoris sp.	YANAGAWA & SAKANISHI, 1980a
		YANAGAWA & SAKANISHI, 1980b
	Lycoris aurea	HUANG & LIU, 1989
	Narcissus sp.	HOL & VAN DER LINDE, 1992
		HANKS & REES, 1977
		HANKS *et al*, 1986
		HANKS, 1986

Amaryllidaceae (cont.)	*Narcissus* sp. (cont)	HANKS, 1987
		HUSSEY, 1982
		HUSSEY & HILTON, 1977
		LANGENS-GERRITS & NASHIMOTO, 1997
	Narcissus bulbocodium	SANTOS *et al.*, 1998
	Narcissus confusus	SELLÉS *et al.*, 1997
		BERGOÑÓN *et al.*, 1996
	Narcissus tazetta	CHEN & ZIV, 2001
		CHEN & ZIV, 2006
		STEINITZ & YAHEL, 1982
	Nerine sp.	MCDONALD, 1985
		PIERIK & IPPEL, 1977
	Nerine angustifolia	CUSTERS & BERGERVOET, 1992
	Nerine bowdenii	GROOTAARTS *et al.*, 1981
		JACOBS *et al.*, 1992
		MOCHTAK, 1989
	Nerine sarniensis	CUSTERS & BERGERVOET, 1992
	Haemanthus sp.	KROMER, 1985
	Hippeastrum hybridum	HUANG *et al*., 1990a
		HUANG *et al.*, 1990b
		HUSSEY, 1975a
		OKUBO *et al*., 1990
		STANCATO *et al.*, 1995
	Hymenocallis sp.	YANAGAWA & SAKANISHI, 1980a
		YANAGAWA & SAKANISHI, 1980b
	Pancraium maritimum	DRAGASSAKI *et al.*, 2003
	Scadoxus sp.	NIEDERWIESER *et al*., 2002
	Sternbergia clusiana	ORAN & FATTASH, 2005
	Vallota purpurea	KUKUŁCZANKA & KROMER, 1988
	Zephyranthes robusata	FURMANOWA & OLEDZKA, 1981
Hyacinthaceae	*Bowiea volubilis*	VAN STADEN *et al.*, 1991
	Drimia robusta	NGUGI *et al.*, 1998
	Eucomis autumnalis	AULT, 1995

Hyacinthaceae (cont.)	*Eucomis comosa*	AULT, 1995
	Eucomis zambesiaca	AULT, 1995
		RAMOGOLA & FENNELL, 2007
	Galtonia candicans	YANAGAWA & SAKANISHI, 1980a
		YANAGAWA & SAKANISHI, 1980b
	Hyacinthus sp.	HUSSEY, 1975b
	Ledebouria graminifolia	SHUSHU *et al.*, 2005
	Muscari racemosum	HUSSEY, 1975a
		KROMER, 1985
		KROMER, 1989
	Ornithogalum sp.	HUSSEY, 1975a
	Ornithogalum saundersiae	KARIUKI, 2008
	Ornithogalum ulphyllum	OZEL *et al.*, 2008
	Ornithogalum umbellatum	NAYAK & SEN, 1995
	Scilla siberica	HUSSEY, 1975a
	Scilla hyacinthoides	YANAGAWA & SAKANISHI, 1980a
		YANAGAWA & SAKANISHI, 1980b
	Urgina indica	JHA *et al.*, 1984
Iridaceae	*Iris* sp.	HUSSEY, 1976
		VAN DER LINDE *et al.*, 1986
		VAN DER LINDE *et al.*, 1988

1.4.3 Tissue culture of the Amaryllidaceae

Many of the Amaryllidaceae have been tissue cultured for the purposes of more rapid propagation. The production of micropropagules of Amaryllidaceae species is increasing (Table 1.1). However they have not been tissue cultured to the extent of other families as the techniques are relatively difficult (VAN DER LINDE, 1992). Tissue culture increases the potential for vegetative reproduction of economically important plants (HUSSEY, 1980). Micropropagation protocols are developed for plants for the purposes of supplying the horticultural trade, the medicinal plant trade, and conserving a plant species.

Chipping, cutting bulbs into segments or 'chips', is one of the methods used to obtain explants from the bulbs of Amaryllidaceae (VAN DER LINDE, 1992). It seems from the literature however that twin-scales are a more popular explant (Table 1.1). More bulbs are produced when scale explants are used as opposed to indirect regeneration from callus (ASCOUGH *et al.*, 2008). Tri-scales and chips are used as explants when twin-scales do not respond to plant growth regulators in culture (FENNELL & VAN STADEN, 2004). It is important that the basal plate is attached to the scales when using twin-scales as explants from Amaryllidaceae. FENNELL and VAN STADEN (2004) report that without the basal plate the explant will not generate bulblets.

Twin-scales were used to establish bulblets in *Crinum variabile* (FENNELL *et al*., 2001). ULRICH *et al*. (1999) successfully established cultures of *Crinum* species from tri-scales. FENNELL and VAN STADEN (2004) report that a number of other *Crinum* species have been propagated through tissue culture.

MORÁN *et al*. (2003) cultured twin-scales from both *Cyrtanthus clavatus* and *Cyrtanthus spiralis* in liquid medium. Shoots were generated from twin-scales. Twin-scales were also the explant of choice in the tissue culture of *Narcissus* 'Golden Harvest' (HOL & VAN DER LINDE, 1992) and *Narcissus bulbocodium* (SANTOS *et al.*, 1998). HUSSEY (1980) generated shoots from twin-scales, leaf bases and the lower part of the stem of *Narcissus*. These explants were attached to a small amount of basal plate tissue.

HUANG and LIU (1989) cultured twin-scales from *Lycoris aurea*. Shoot-tip explants of *Lycoris aurea* were also cultured, however these cultures did not develop as well as the twin-scale explants. DREWES and VAN STADEN (1994) generated bulbs *in vitro* from bulb scale explants of *Gethyllis linearis*. Leaf, scale and stem explants may be used to generate shoots of *Nerine* in culture (HUSSEY, 1980). *Agapanthus, Altromeria, Amaryllis, Clivia, Furcraea* and *Galanthus* are members of the Amaryllidaceae which have been successfully propagated *in vitro* (FINNIE, 1989).

The growth and development of an explant in culture depends on four factors: (1) the genetic "make-up" of the plant; (2) the nutrients (water, macro- and micro-elements and sugars) available to the explant; (3) environmental factors (light, temperature, pH, O_2 and CO_2 concentrations); and (4) organic substances (growth regulators and vitamins) available to the explant (PAN & VAN STADEN, 1998).

1.4.4 Tissue culture of *Brunsvigia* species

The literature does not describe the tissue culture of any *Brunsvigia* species indicating that there have not been any successful attempts to tissue culture any plants in this genus. "Biotechnological approaches are used to meet the growing demand for bulbs and to conserve those over-exploited by the herbal medicine trade" (FENNELL & VAN STADEN, 2004).

1.4.5 The effect of nutrients in culture

MURASHIGE & SKOOG, (1962) (MS) is the most common basal medium used in the tissue culture of the Amaryllidaceae (GEORGE, 1993). MS basal medium contains both macro-nutrients and micro-nutrients (Table 1.2), sugars (*myo*-inositol and sucrose) and in the case of solid medium, a gelling agent (Agar or Gelrite).

Sucrose concentration can have a marked effect on organogenesis in culture. High sucrose concentrations can induce bulbing from shoots in *Cyrtanthus* species (MORÁN *et al*., 2003). Bulblet size increased as sucrose concentration increased in *Lilium longiflorum* and *Cyrtanthus* species (MORÁN *et al*., 2003; HAN *et al*., 2005).

In *Ornithogalum maculatum* high sucrose concentrations induced bulblet formation while low sucrose concentrations resulted in the formation of shoots (VAN RENSBURG *et al*., 1988). SANTOS *et al*. (1998) showed that the optimum sucrose concentration for bulblet formation in *Narcissus bulbocodium* is 9%. Alternatively, the best bulblet formation was obtained with a sucrose concentration of 3% in *Lilium* species (VARSHNEY *et al*., 2000). The effects of sucrose concentration are species and explant specific.

Table 1.2: The components of a modified MURASHIGE and SKOOG (1962) medium.

Components	**Salts**	**mgl^{-1}**	**mll^{-1}**
Macro nutrients	NH_4NO_3	1650	10
	KNO_3	1900	20
	$CaCl_2.2H_2O$	440	10
	$MgSO_4.7H_2O$	370	10
	NaFeEDTA	37.3	10
	KH_2PO_4	170	10
Micro nutrients	H_3BO_3	6.2	10
	$MnSO_4.4H_2O$	22.3	
	$ZnSO_4.7H_2O$	8.6	
	KI	0.84	
	$Na_2MoO_4.2H_2O$	0.25	10
	$CuSO_4.5H_2O$	0.025	
	$CoCl_2.6H_2O$	0.025	
Vitamins	Thiamine HCl	0.1	10
	Nicotinic acid	0.5	
	Pyridoxin HCl	0.5	
	Glycine	2	

1.4.6 The effect of environmental factors on tissue culture

Environmental factors such as light and temperature are important factors in the success or failure of plant tissue culture systems. The photoperiod at which plant cultures are kept will have an effect on how they respond in culture. Light plays a key role in the everyday life of plants and so has to be considered in a plant tissue culture protocol. The light regime may differ in the intensity of light and the number of hours of light (photoperiod) that the explant is subjected to. The optimum photoperiod will be species specific and is often dependent on the light conditions under which the species grows in the wild.

Bulblets which develop in the dark differ from those which develop in the light (LESHEM *et al*., 1982). *Nerine bowdenii* twin-scales produced more bulblets per explant with greater fresh weights when cultured in the light than in the dark (JACOBS *et al*., 1992). Conversely, STEINITZ and YAHEL (1982) found that fewer bulblets per explant with a lower fresh weight were produced in cultures in the light. Light can have either a stimulatory and inhibitory effect on plant cultures.

The optimum temperature at which to keep cultures will differ among species, although most species are cultured at 25 °C. Bulbous species often require temperatures lower than 25 °C (YEOMAN, 1986).

1.4.7 The effect of organic substance concentration on the Amaryllidaceae

Auxins and cytokinins are the two types of plant hormones which are most often incorporated into the tissue culture media of Amaryllidaceae. These are the most critical plant growth regulators (FENNELL & VAN STADEN, 2004). Varying ratios of auxin to cytokinin have been examined for their effect on amaryllids *in vitro*. The effect of hormones *in vitro* will depend on the internal hormone concentrations of the explant.

FENNELL and VAN STADEN (2004) state that species of the Amaryllidaceae need higher cytokinin concentrations than other families to promote branching. FENNELL *et al*. (2001) found that *Crinum variable* required the cytokinin, benzyladenine (BA) without the auxin, α-naphthalene acetic acid (NAA) for shoot outgrowth.

HUANG and LIU (1989) found that an equal ratio of NAA to BA induced the greatest shoot production in *Lycoris aurea*. However, a high BA to NAA ratio showed optimal normal shoot development. The same was found for the multiplication of shoots. During the rooting of shoots, however, the greatest success was recorded for shoots in media that were without BA, but supplemented with NAA. Similar patterns in optimum hormone concentrations were observed for *Narcissus bulbocodium* (SANTOS *et al.*, 1998) and *Cyrtanthus clavatus* and *Cyrtanthus spiralis* (MORÁN *et al.*, 2003). While ULRICH *et al*. (1999) reported that a medium with a high cytokinin concentration and devoid of auxin showed the greatest proliferation of shoots.

Activated charcoal is another organic substance that can be incorporated into tissue culture media. Activated charcoal has the ability to adsorb inhibitory substances in the medium (PAN & VAN STADEN, 1998; FENNELL & VAN STADEN, 2004). Inhibitory substances such as phenolics are often released by plant materials in culture (PAN & VAN STADEN, 1998). These can be toxic to explants (WANG & HUANG, 1976) and can be adsorbed by activated charcoal.

When added to the medium, activated charcoal darkens the medium (WEATHERHEAD *et al.*, 1978; PAN & VAN STADEN, 1998), which resembles soil conditions. This is beneficial when cultures are initiated using bulb explants as they are subjected to continuous dark in the wild.

1.5 AIMS

The aim of this study was to develop a protocol for the tissue culture of *Brunsvigia undulata*, thereby creating a means for the mass production of these bulbs. This will decrease the pressure on this species in the wild from plant collectors and overuse by traditional healers.

Chapter 2

TISSUE CULTURE FROM SEEDS

INTRODUCTION

Most plant cells are totipotent, they hold embryogenic competence (FENNELL, 2002). This ability allows explants from many different plant tissues to regenerate plantlets (VAN AARTRIJK & VAN DER LINDE, 1986). Not all explants will form meristematic structures at the same frequency. Also these structures may not form on all parts of an explant (BOONEKAMP, 1997).

The implementation of tissue culture in the propagation of bulbous plants has a number of advantages. One of these is that it allows for many clonal plants to be produced relatively quickly. The natural rate of vegetative propagation of bulbous plants, in particular the Amaryllidaceae is low (COLQUE *et al*., 2002; SANTOS *et al*., 2002) and conventional propagation methods are labour intensive (SANTOS *et al*., 2002).

HUSSEY (1986) explains that *in vitro* propagation is achieved by the following procedures: (1) explant selection, the selection of small pieces of growing plants, their decontamination and establishment of cultures on a growth medium; (2) the subculture onto a medium to promote induction or proliferation of shoots; and (3) rooting of the shoots and planting out in the greenhouse or the field. These three steps form the basis of the work reported in this Chapter.

MATERIALS AND METHODS

An overview of the methods used to establish and multiply propagules of *Brunsvigia undulata* from seed is presented by Figure 2.1 (see p. 54).

Seeds were collected (A) and 20 seeds were germinated on each four different media strengths, $^{1}/_{10}$, ½, ¼, and full strength (B). Seedlings grown *in vitro* were dissected into

sections and sub-cultured (C). Seedling sections formed callus and bulblets (D) which were sub-cultured (E). Callus formed shoot clusters and bulblets rooted (F). Shoot clusters were separated and individual shoots were placed onto rooting media (G). Callus from shoot clusters was placed back onto hormone-free media and continued to form shoot clusters (F & G). Shoots on four different rooting media formed roots (H). Rooted bulblets were planted out in a 1:1 vermiculite: perlite potting medium and placed in a mist house (I).

Unless otherwise stated the growth medium used was MS (MURASHIGE & SKOOG, 1962) with 0.1 gl^{-1} *myo*-inositol, 8 gl^{-1} agar (Agar Bacteriological-Agar No. 1, Oxoid Ltd., England) and 3% sucrose (w/v). The culture medium was sterilized by autoclaving for 20 min at 121 °C and 103.4 kPa.

Explants were placed on 10 ml of medium in 40 ml culture tubes which were sealed with metal caps and a 1 cm thick strip of Parafilm®. Cultures were kept at 25 ± 1 °C under Osram® 75 W cool white fluorescent tubes in a 16 h photoperiod with a light intensity of 34.2 µmol m^{-2} s^{-1}.

Where results were recorded as percentages the data were arcsine transformed prior to statistical evaluation (SCOTT *et al.*, 1984). An analysis of variance (ANOVA) was carried out on all data. Data were analysed using a Duncan's test at the 5% level in SPSS (Statistical Package for the Social Sciences), version 10.0.

2.1 PLANT COLLECTION AND DECONTAMINATION

Introduction

The seeds of *Brunsvigia undulata* F. M. Leight., are produced in capsules at the ends of dried flower stalks (Figure 1.2 B and Figure 1.3 A). Seeds were collected when capsules were about to burst. Once seeds had been collected they could not be stored and so they were put into culture almost immediately.

Figure 2.1 A-E

Figure 2.1 F-I

Decontamination is of utmost importance when developing a protocol for the tissue culture of a plant species. If a successful decontamination protocol is not first developed, cultures cannot be established. Bacteria, viruses, and even mites can cause the loss of cultures and will effect the growth and morphogenesis of explants. DEBERGH and MAENE (1981) believe that avoiding contamination is of primary concern when establishing tissue cultures.

There are a number of different decontaminating agents which can be used. These include fungicides, antibiotics and other biocides (FENNELL, 2002). The most widely used sterilants are solutions of sodium or calcium hypochlorite and mercuric chloride. Sodium hypochlorite is available as commercial bleach and often plant parts need an exposure to the decontaminating solution of up to 25 min for all contaminants to be removed (SWEET & BOLTON, 1979). Mercuric chloride is very effective for decontaminating plant parts. A high percentage decontamination may be achieved by using mercuric chloride in combination with a fungicide (LEIFFERT & WAITES, 1994). Although highly effective mercuric chloride is toxic (FENNELL, 2002) not environmentally friendly due to its heavy metal content. For this reason non-toxic alternatives such as sodium or calcium hypochlorite or 70% ethanol (SWEET & BOLTON, 1979) should be used.

Seeds need only be surface decontaminated. As a seed is a sealed entity, it is hoped that the inside of the seed is free from contaminants. However, this is not always the case.

Materials and Methods

Seeds were collected, at the beginning of March, from plants growing along the road to Mount Gilboa (29° 16.764′ S, 30° 17.627′ E) and at Wharoonga (29° 36.588′ S, 30° 08.016′ E). Half of the seeds collected were sown in a potting mixture, comprising of 83% compost, 16.72% bark, 0.14% LAN and 0.14% 2:3:2 fertilizer. Trays containing seeds were kept under shade cloth in the botanical gardens at the University of KwaZulu-Natal and were watered daily.

The remaining seeds were washed well with tap water to remove any excess dirt and subsequently decontaminated in 0.2% mercuric chloride with a few drops of Tween 20 for 5 min. Three changes of sterile distilled water were used to rinse the mercuric chloride off the seeds.

Results

Seed decontamination was 100% successful. There was no contamination by bacteria or fungi and so there was no loss of cultures.

Discussion

Mercuric chloride is a potent decontaminating agent. This will account for the success in decontamination of the seeds before they were put into culture. As seeds are assumed to be relatively sterile inside, surface sterilization is mostly sufficient when decontaminating seeds. Mercuric chloride is an effective decontaminating agent for the decontamination of *Brunsvigia undulata* seeds.

Mercuric chloride has a high heavy metal content which can cause damage to plant material (FENNELL, 2002). This decontaminating agent also has to be disposed of very carefully as it is not environmentally friendly. It would therefore be preferable to use a safer alternative such as sodium or calcium hypochlorite. As seeds of *Brunsvigia undulata* were only available once during this study it was important that seed decontamination was successful. For this reason the strongest decontaminating agent available was used.

2.2 CULTURE ESTABLISHMENT AND SEED GERMINATION

Introduction

Brunsvigia undulata produce large, fleshy green seeds (Figure 1.3 A). The seeds of *Brunsvigia undulata*, akin to other members of the Amaryllidaceae family, are recalcitrant. Recalcitrant seeds have the ability to germinate and establish themselves quickly after they have fallen from the plant (COPELAND & MCDONALD, 2001). They do not go into dormancy, but rather continue to develop and germinate (BERJAK *et al.*, 1990).

Recalcitrant seeds have a high moisture content, between 50% and 70% at maturity (COPELAND & MCDONALD, 2001) and so they will germinate without being imbibed. This allows for the seeds to germinate readily without water and in the driest conditions (VERDOORN, 1973). A number of the *Brunsvigia undulata* seeds collected for this study germinated in the brown paper bag that they were collected in. The seeds of *Brunsvigia* species have a short-lived viability (OLIVER, 1990). If they are not sown while they are still green they will not germinate.

In vitro germination is often carried out on media with a low concentration of salts and little or no sucrose. High salt concentrations in the germination media will cause the seed to loose water during imbibtion (HARDEGREE & EMMERICH, 1990). This will prevent the seed from germinating. Sucrose in the germination media will inhibit protein activation and mobilization within the seed (BOREK & RATAJCZAK, 2002). The effect of salt concentration on seed germination was examined in this experiment and no sucrose was used in the germination media.

Materials and methods

Media for the germination experiment did not include sucrose. The culture conditions were as stated in the Materials and Methods on page 22. Twenty seeds were placed on

each treatment ($^1/_{10}$, $^1/_2$, $^1/_4$, and full strength MS). Cultures were checked daily and germination was recorded. Mean germination time (MGT) was calculated using the equation: MGT = $\Sigma(n \times d)/N$, where n = number of seeds germinated on each day, d = number of days from the beginning of the experiment, and N = total number of seeds germinated at the end of the experiment (ELLIS & ROBERTS, 1981).

Results

The results of the germination experiment are shown in the Figures 2.2 and 2.3.

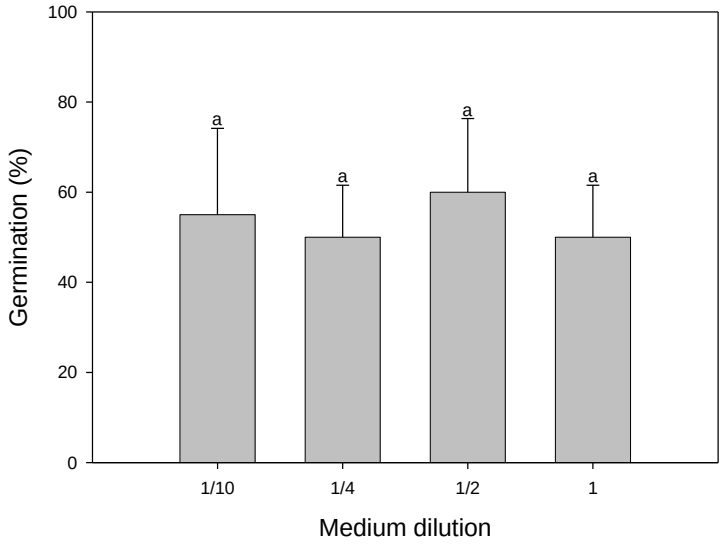


Figure 2.2: The effect of medium concentration on percentage seed germination. Bars represent standard error. Different letters indicate significant differences between treatments at the 5% level (ANOVA).

Figure 2.2 shows that the highest germination was achieved on ½ strength MS (60% germination). The differences between germination percentages in the different treatments were not significant.

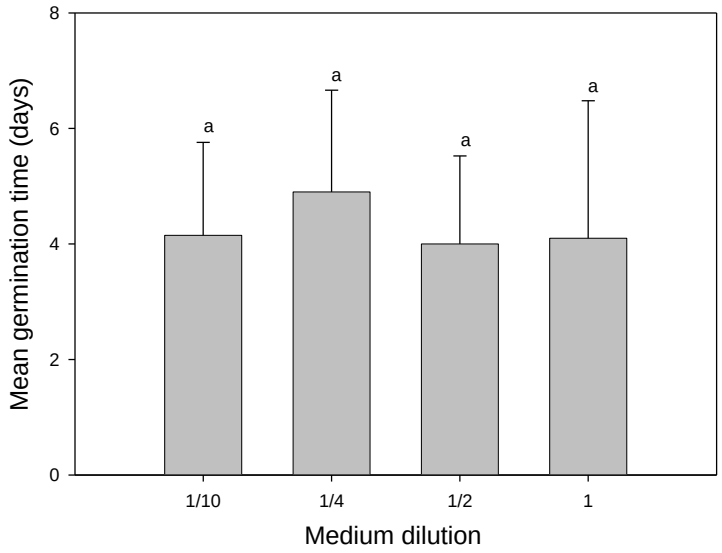


Figure 2.3: The effect of medium concentration on the number of days taken for seeds to germinate. Bars represent standard error. Different letters indicate significant differences between treatments at the 5% level (ANOVA).

Figure 2.3 shows that seeds on ½ strength MS were the quickest to germinate, on average the seeds in this treatment germinated within 4 days. Although there were slight differences, the seeds in each treatment in general, germinated on their fourth day in culture. The MGT of the seeds in the different treatments were not statistically significant.

Discussion

Figure 2.2 indicates that seeds placed on all four media concentrations had fairly similar germination percentages. Although there were slight differences between the different media concentrations these were not statistically significant. Germination and seedling development took place with all media concentrations.

The recalcitrant nature of *Brunsvigia undulata* seeds means that they germinate soon after they are shed from the mother plant. They possess little or no dormancy capacity

(COPELAND & MCDONALD, 2001). The seeds contain all that is needed to germinate when shed from the mother plant. This allows them to germinate successfully under a variety of conditions. The seeds probably did not require any nutrients from the growth medium and so the concentration of the medium did not have an effect on germination.

Seeds on the $^1/_2$ strength media were quickest to germinate. The seeds on $^1/_{10}$ strength and full strength MS took slightly longer to germinate while those on $^1/_4$ strength MS were the slowest to germinate (Figure 2.3). These differences, however, were not statistically significant. The size and developmental status of the seed may have had an effect on how long the seed took to germinate.

Seeds which were placed on the stronger MS medium may have lost water (HARDEGREE & EMMERICH, 1990). For seeds of many species this loss of water would have been detrimental and they would not have been able to germinate. *Brunsvigia undulata* seeds, however, are recalcitrant and have a very high water content. They can afford to loose water to a degree, and so they were able to germinate on media with a high concentration of salts.

Medium concentration did not effect the germination, with regards to final percentage germination or germination rate, of *Brunsvigia undulata* seeds *in vitro*.

2.3 CULTURE OF SEEDLING SECTIONS

Introduction

Once the seeds had germinated *in vitro*, the developed seedlings which formed can be used as a source of explants for further experimentation. There are advantages to using *in vitro* grown seedlings. A single seedling provides explants from more than one of its component parts as explants can be obtained from roots, coleoptiles (or hypocotyl) and cotyledons of the seedlings.

Seedlings grown aseptically from decontaminated seed are a good source of shoot-tips and young organ explants for further cultures (HUSSEY, 1986). Shoot multiplication was obtained for *Withania somnifera* from shoot-tips of seedlings germinated *in vitro* (SEN & SHARMA, 1991). These explants will not have any damage from sterilants (HUSSEY, 1986) as only the seed coat came into contact with the sterilant during decontamination.

Sections excised from aseptically grown seedlings have been reported to develop meristematic tissue and callus. All sections excised from seedlings of *Babiana* species formed meristematic tissue (JÄGER *et al*., 1995). KUKUŁCZANKA *et al*. (1989), obtained bulblets from seed explants of *Fritillaria meleagris*. Plantlets were also obtained directly from seedling explants of *Gloriosa* (FINNIE & VAN STADEN, 1989). Callus which formed on seedling sections of *Phaseolus vulgaris* was used to initiate further cultures (GÉMESNÉ *et al*., 1997). Leaf explants from seedlings of *Ornithogalum maculatum* formed shoots, from callus formed at explant edges, when cultured *in vitro* (VAN RENSBURG *et al*., 1988).

Materials and methods

Once seeds had germinated and seedlings had developed, the seedlings were divided into ten sections (Figure 2.4). Ten of each section were put onto the sterilized MS in each treatment. The media were supplemented with combinations of 0, 0.5, 1 or 2.5 mgl^{-1} BA and 0, 0.5, 1, 2.5 mgl^{-1} NAA. The culture conditions were as stated in the Materials and Methods on page 22.

Sections 1, 2 and 3 were excised from the primary roots of the seedlings. Section 4 was the junction between the root and shoot. Section 5 was the area of coleoptile before the cotyledons. Section 6 was the base of the cotyledons and top of the coleoptile. Sections 7, 8, 9 and 10 were excised from the cotyledons of the seedlings.

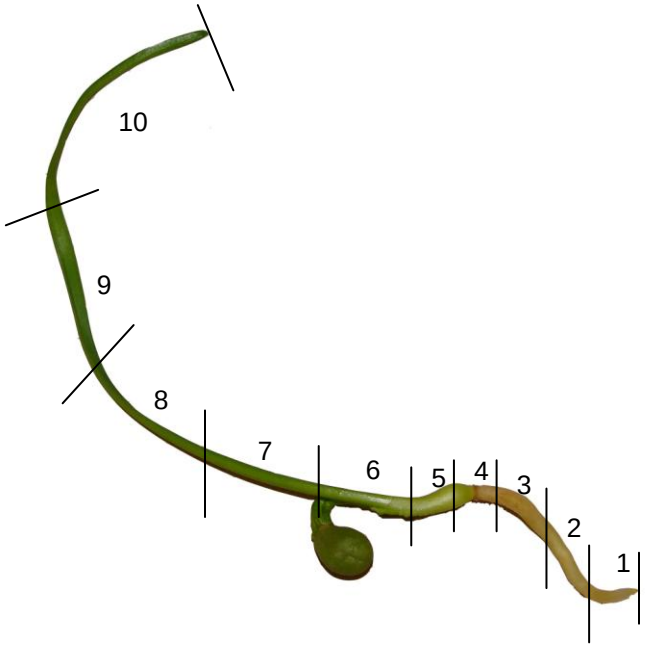


Figure 2.4: ***Brunsvigia undulata*** **seedling showing the ten Sections (1-10) into which seedlings were divided.**

Results

Explants from seedlings produced roots, shoots, bulblets and callus. These structures formed on various parts of the explants. There was no consistency as to where on the explant these structures formed.

Table 2.1: Growth response of seedling sections on media supplemented with BA and NAA. The areas highlighted show structures which were used for subsequent experiments.

Seedling Section	BA:NAA ratio (mgl^{-1}) 0:0	0.5:0	0.5:2.5	1:1	2.5:0.5	0:0.5
1	Elongation	Elongation	Swelled, Multiplication	Elongation, Multiplication, Callus	Elongation, Swelled, Callus	Elongation, Multiplication
2	Swelled	Swelled	Swelled, Multiplication	Multiplication, Callus	Elongation, Swelled, Callus	Swelled, Multiplication
3	Swelled	Swelled	Swelled	Swelled	Callus	Swelled, Multiplication
4	Shoots, Roots, Bulblets	Shoots, Roots, Bulblets	Shoots, Roots, Bulblets	Shoots, Roots, Bulblets	Shoots, Roots, Callus	Shoots, Roots, Callus
5	Swelled	x	Roots, Bulblets, Callus	Bulblets, Callus	Bulblets, Callus	Callus
6	x	x	Shoots, Roots, Callus	Bulblets, Callus	Bulblets, Callus	Bulblets
7	x	x	x	x	Shoots, Roots, Callus	x
8	x	x	x	x	x	x
9	x	x	x	x	x	x
10	x	x	x	x	x	x

Sections 1, 2 and 3, which were excised from the roots of seedlings, either grew in length or formed further roots or callus. These sections did not, however, form meristematic structures. Sections 4, 5, 6, and 7 formed meristematic structures. Section 4 formed shoots, roots and bulblets on four treatments. Bulblets were formed by Section 5 explants on only three of the treatments. Section 6 explants also only formed bulblets on three of

the treatments. Shoots, roots and callus were only formed by Section 7 explants on the 2.5:0.5 mgl^{-1} BA: NAA treatment. Sections 8, 9 and 10 did not respond in culture.

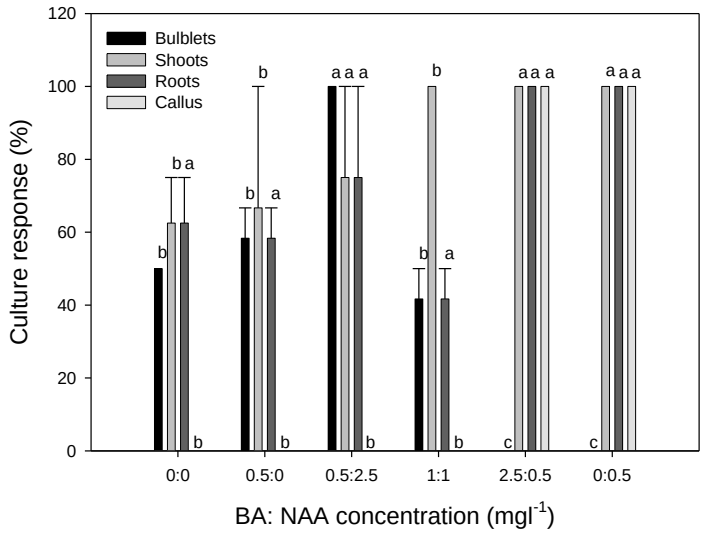


Figure 2.5: The percentage bulblet, shoot, root and callus induction by Section 4 explants on medium supplemented with different concentrations of BA and NAA. Bars indicate standard error. Different letters indicate significant differences between treatments at the 5% level (ANOVA).

Figure 2.5 shows the percentage of Section 4 explants which produced bulblets, shoots, roots and callus when cultured on MS supplemented with different concentrations of BA and NAA. Highest bulblet production was obtained by explants on medium supplemented with 0.5 mgl^{-1} BA and 2.5 mgl^{-1} NAA. All the explants on this medium formed bulblets. Bulblets formed at a lower frequency on all other treatments, except two treatments where bulblets did not form.

All the explants on medium supplemented with 1 mgl^{-1} BA and 1 mgl^{-1} NAA formed shoots. All explants on 2.5 mgl^{-1} BA and 0.5 mgl^{-1} NAA and 0.5 mgl^{-1} NAA without BA, produced shoots, roots and callus. Roots formed in all treatments. Callus was only formed in two treatments.

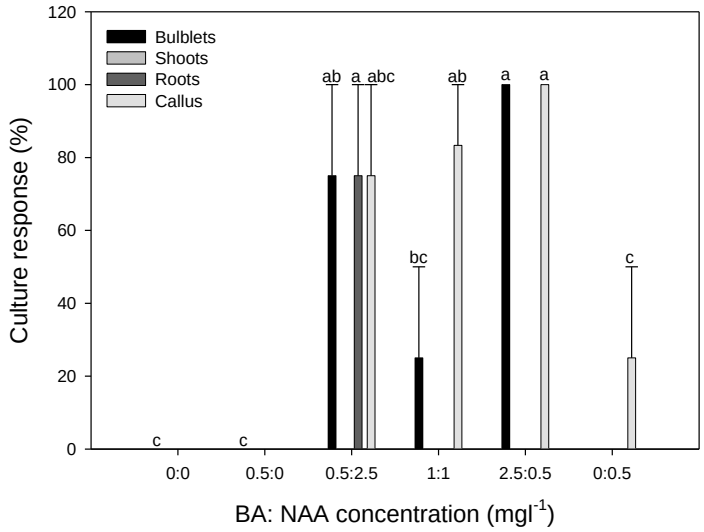


Figure 2.6: The percentage bulblet, shoot, root and callus induction by Section 5 explants on media supplemented with different concentrations of BA and NAA. Bars indicate standard error. Different letters indicate significant differences between treatments at the 5% level (ANOVA).

Figure 2.6 shows the percentage of Section 5 explants which produced bulblets, shoots, roots and callus when cultured on media supplemented with different concentrations of BA and NAA. Explants cultured on media without hormones and on media supplemented with 0.5 mgl^{-1} BA without NAA did not form bulblets, shoots, roots or callus. Bulblets were produced by explants in three of the treatments. Bulblets formed on 2.5 mgl^{-1} BA and 0.5 mgl^{-1} NAA (100%), 0.5 mgl^{-1} BA and 2.5 mgl^{-1} NAA (75%) and 1 mgl^{-1} BA and 1 mgl^{-1} NAA (20%).

Shoots were not produced with any of the treatments. Roots were only produced by one treatment (0.5 mgl^{-1} BA and 2.5 mgl^{-1} NAA). Callus formed on explants in four of the treatments.

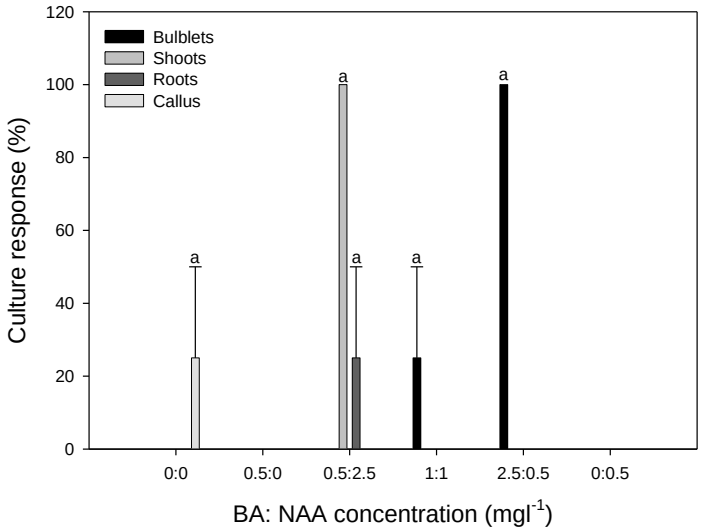


Figure 2.7: The percentage bulblet, shoot, root and callus induction by Section 6 explants on media supplemented with different concentrations of BA and NAA. Bars indicate standard error. Different letters indicate significant differences between treatments at the 5% level (ANOVA).

Figure 2.7 shows the percentage of Section 6 explants which produced bulblets, shoots, roots and callus when cultured on media supplemented with different concentrations of BA and NAA. Explants cultured on medium without hormones and on medium supplemented with 0.5 mgl^{-1} BA and without NAA did not form bulblets, shoots or roots. Bulblets were formed on explants in two treatments, on medium supplemented with 1 mgl^{-1} BA and 1 mgl^{-1} NAA and on medium supplemented with 2.5 mgl^{-1} BA and 0.5 mgl^{-1} NAA.

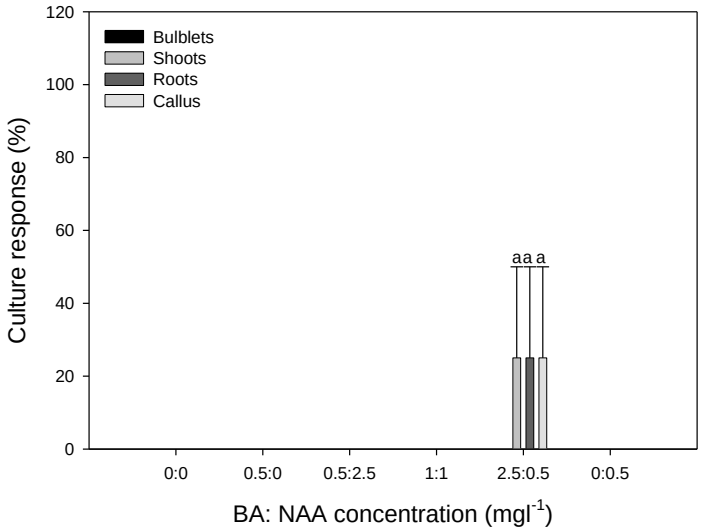


Figure 2.8: The percentage bulblet, root, shoot and callus induction by Section 7 explants on media supplemented with different concentrations of BA and NAA. Bars indicate standard error. Different letters indicate significant differences between treatments at the 5% level (ANOVA).

Figure 2.8 shows the percentage of Section 7 explants which produced bulblets, shoots, roots and callus when cultured on media supplemented with different concentrations of BA and NAA. Only explants on medium supplemented with 2.5 mgl^{-1} BA and 0.5 mgl^{-1} NAA formed shoots, roots and callus. Shoots, roots and callus were produced by 33% of explants in this treatment. There were no statistically significant differences with organ induction for Section 7 explants.

Discussion

Different plant tissues differ in their ability to undergo morphogenesis and form meristematic structures (FENNELL, 2002). Sections 1 to 10 did not all undergo morphogenesis in culture. Those that did, however, formed similar meristematic structures, at different frequencies. Not all explants will form meristematic structures at the same frequency (BOONEKAMP, 1997; FENNELL & VAN STADEN, 2004). Meristematic regions are usually the most successful explants in tissue culture (FENNELL, 2002).

Root explants (Sections 1, 2 and 3) only produced further root tissue and callus. Root tissue from seedlings of *Babiana* species, produced bulblet-like structures (JÄGER *et al*., 1995). Without the correct hormones root cells cannot be induced to form different plant structures. Callus was formed by all three root sections in at least one of the treatments (Table 2.1). However, the callus was fragile and broke up when it was sub-cultured.

Section 4 was the most productive of the sections in culture. This section includes the junction between the root and coleoptile of the seedling. At this junction is the meristem. This explains the formation of bulblets, shoots, roots and callus by this section (Table 2.1). A pre-existing meristematic region in the explant, supersedes the need for cells to become meristematic before morphogenesis can occur.

Bulblets were not produced in the presence of high BA concentrations (Figure 2.5). Nor were they formed when only NAA was present at low concentrations in the media. The greatest frequency of bulblet formation was obtained when there was a 1:5 ratio of BA:NAA. When compared to the control, bulblet production in this treatment was 100% greater. High concentrations of BA resulted in the production of callus. Callus was also produced in the presence of low concentrations of NAA and the absence of BA. As callus and bulblets were sub-cultured for multiplication, Section 4 was most successful on medium supplemented with 0.5 mgl^{-1} BA and 2.5 mgl^{-1} NAA. Shoots formed on Section 4 explants in all treatments. Shoot production was greater when NAA was present in the medium.

Sections 5, 6 and 7 did not include a meristematic region. The lack of meristematic cells in the explant prevents morphogenesis from taking place until cells in the explant become meristematic. This will account for the difference in regeneration rate and ability of these explants when compared to explants from Section 4 of the seedlings.

Section 5 was comprised only of coleoptile tissue. JÄGER *et al.* (1995) found that hypocotyl segments from *Babiana* species formed meristematic structures at a higher

frequency than root and leaf explants. This section did not show any organogenesis in the absence of NAA (Figure 2.6). Bulblets were not produced in the absence of BA. All explants formed bulblets and callus when BA and NAA were present at a ratio of 5:1. Bulblets and callus were also formed when BA and NAA were present at a ratio of 1:5, however only 75% of explants formed bulblets and callus. Both BA and NAA must be present in the medium for bulblets to form. Adventitious meristems may be induced directly on explants such as stem sections. This however usually requires the presence of auxin, or both auxin and cytokinin (HUSSEY, 1980). Callus did not form in the absence of NAA, and the largest number of explants produced callus when BA and NAA were present in the medium at a ratio of 5:1.

Section 6 showed poor bulblet production. Bulblets were only produced by explants on two treatments, when BA and NAA were present at equal concentrations and when only NAA was present in the media (Figure 2.7).

Section 7 only produced meristematic tissue in one treatment (Figure 2.8). All explants formed callus and shoots when BA and NAA were present in the medium at a ratio of 5:1. Cotyledon explants from *Brunsvigia undulata* seedlings require a high ratio of BA to NAA to form callus.

Explants which did not contain the meristematic region between the root and the coleoptile (Sections 5, 6 and 7), did not show organogenesis in the absence of NAA. Callus production was greatest when both BA and NAA were present in the media

In conclusion, the junction between the root and shoot (Section 4) is the most productive seedling section. This section readily forms bulblets, shoots and callus, which may be used for subsequent culture establishment.

2.4 MULTIPLICATION FROM BULBLETS, SHOOTS AND CALLUS

Introduction

Multiplication of propagules can be obtained through one of two tissue culture pathways. The first is through shoot clumps (BERGOÑÓN *et al.*, 1992; 1996). Shoots form from the base of shoot clumps. The base of the shoot clump consists of amorphous achlorophyllous tissue (FENNELL, 2002) (Figure 2.9). This tissue has a structure similar to that of the basal plate of a bulb (CHOW *et al*., 1993). Secondly, the formation of storage organs (e.g. bulblets) can be induced (GEORGE, 1993). Storage organs are then used to initiate further cultures. Callus in not often used, although it can be induced to form adventitious shoots (FENNELL, 2002). Callus is however more easy to manipulate at different developmental stages than other propagules (ZIV *et al*., 1995).

Shoots of *Narcissus* formed between 10 and 12 adventitious shoots on hormone-free media (BERGOÑÓN *et al*., 1992). Bulblets and shoots transferred to hormone-free media may root, depending on the species. Auxin is usually required in the media to induce rooting although some species will root without hormones. HUSSEY (1980) found that rooting generally occurred for *Narcissus* and *Nerine* when shoots were transferred to hormone-free media. Rooting of *Withania somnifera* shoots from seedling shoot tips was successful on hormone-free media (SEN & SHARMA, 1991).

Callus can behave in different ways when sub-cultured. The callus can grow in size, continuing to produce undifferentiated cells (GÉMESNÉ *et al*., 1997). Callus can become photosynthetic. On the other hand the cells can differentiate and form organs, such as shoots, roots and bulblets. Shoot formation takes place when pieces of callus are transferred to media containing little or no hormones (HUSSEY, 1980).

Materials and methods

Callus, bulblets and shoots produced by seedling sections were cultured on hormone-free MS. The culture conditions were as stated in the Materials and Methods on page 22.

Results

Multiple shoots were formed by callus explants alone. Bulblets and shoots did not multiply. Shoots developed bulblets at their bases. Bulblets rooted and grew in size. Callus from the seedling sections formed shoot clusters (Figure 2.9). An average of 10.1 ±1.2 shoots were produced by callus explants. Shoots were green and healthy. The arrow shows shoots forming from the achlorophyllous tissue at the base of the shoot clump.

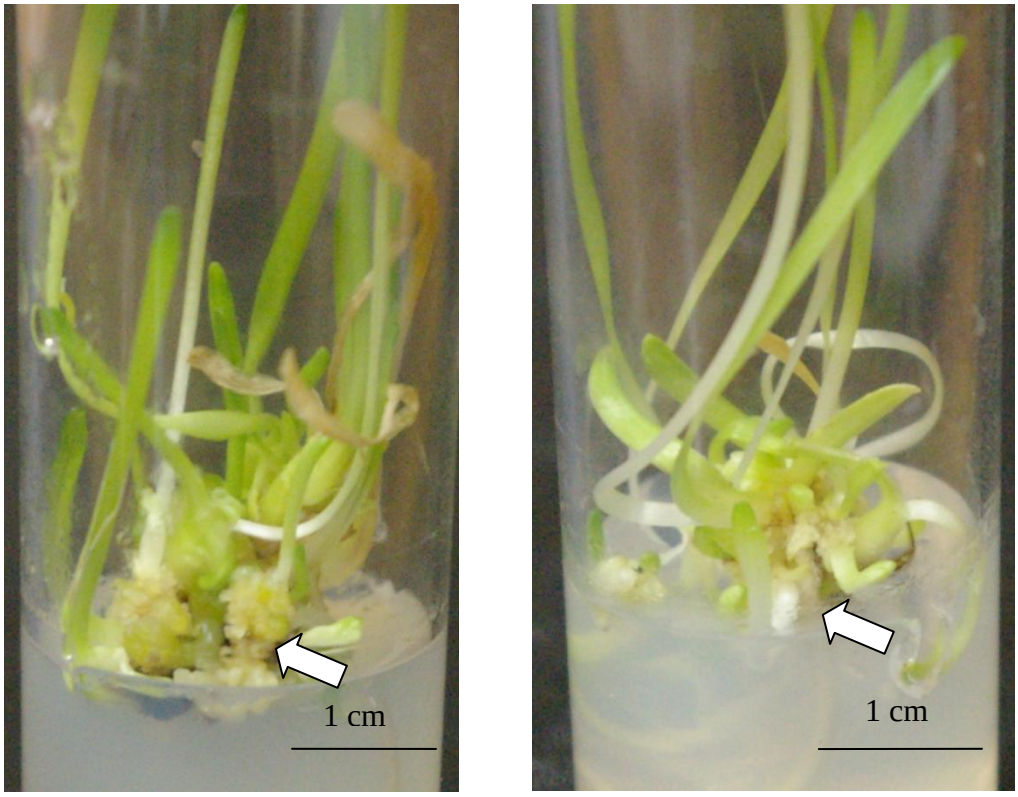


Figure 2.9: Shoot clusters formed by callus on hormone-free MS medium. The arrows indicated the base of the shoot cluster.

All bulblets and shoots from seedling sections grew larger and produced roots and shoots (Figure 2.10).

Figure 2.10: Bulblets from seedling sections. Bulblets grew in size, producing roots and shoots.

Discussion

Callus produced by seedling sections did not require growth hormones to produce shoot clusters. Multiplication from callus was prolific. An average of 10.1 shoots were produced by each callus explant. Shoot production of the same magnitude was reported

by BERGOÑÓN *et al.* (1992) for *Narcissus* where shoot clumps comprised of 10-12 shoots.

Similarly, bulblets and shoots produced by seedling sections did not require plant growth hormones to develop and root. Shoots produced healthy bulblets and roots. Bulblets grew larger and also produced roots. Shoots of *Allium ascalonicum* produced *in vitro* rooted spontaneously on hormone-free media (BERLJAK & BENEDICIC, 1997). Bulblets of *Crinum moorei* did not need a rooting stage before acclimatization as they formed roots spontaneously (FENNELL, 2002). As bulblets and shoots from seedling sections rooted on this media there was no need to transfer them to rooting media. They could be taken through to the acclimatization stage.

2.5 ROOTING AND SECONDARY MULTIPLICATION

Introduction

Shoot clusters can be used to continue multiplication of shoots. Shoot clusters can be dissected into several parts and sub-cultured (KIM & DE HERTOGH, 1997). Divided shoot clusters will usually continue to generate further shoots.

Rooting will enable bulblets to establish successful *ex vitro* (FENNELL & VAN STADEN, 2004). However, bulblets do not need a rooting stage before they are transferred to the natural environment as they may be sown like seed (FENNELL *et al*., 2001).

The plant growth hormone auxin enhances *in vitro* shoots or bulblets to root (KIM & DE HERTOGH, 1997; FENNELL & VAN STADEN, 2004). While cytokinins are not commonly used in rooting media as they can inhibit rooting in the Amaryllidaceae (ANGULO *et al*., 2003). The rooting of *Narcissus* bulblets was higher in the presence IBA than in the presence of NAA (SANTOS *et al*., 2002) and root formation was greater in *Cucumis sativus* shoots in the presence of IBA than in the presence of NAA

(SELVARAJ *et al*., 2007). IBA was the auxin used to induce rooting for shoots of *Scilla siberica* (CHAUDHURI & SEN, 2002), bulblets of *Allium ampeloprasum* (ZIV *et al*., 1983) and bulblets of *Eucharis grandiflora* (PIERIK *et al*., 1983). NAA was the auxin of choice for rooting bulblets of *Eucrosia stricklandii* (COLQUE *et al*., 2002) and bulblets of *Narcissus tazetta* (STEINITZ & YAHEL, 1982).

Rooting occurred without plant growth regulators in *Gladiolus* (REMOTTI & LÖFFLER, 1995), *Crinum moorei* (FENNELL, 2002), and *Babiana* (JÄGER *et al*., 1995).

The induction of callus to form adventitious buds is an effective way of multiplying shoots and bulblets (KIM & DE HERTOGH, 1997). Callus can form adventitious buds which grow into shoots or even bulblets.

Materials and methods

The media were supplemented with 0.1, 1 and 10 mgl^{-1} IBA. Shoot clusters, resulting from the callus from seedling sections which had been in culture for 60 days, were separated out into individual shoots. Individual shoots were cultured on the three rooting media.

The callus which was left behind after shoot clusters had been separated was dissected into 5 x 5 mm explants and cultured on MS medium. These callus cultures produced further shoot clusters which were separated out again and sub-cultured as described above.

Further shoots were placed on MS medium without hormones. This treatment acted as a control for the rooting experiment and also allowed for further multiplication from the shoots. The culture conditions were as stated in the Materials and Methods on page 22.

Results

Shoots formed bulblets, which in turn formed roots in culture. The results of the above mentioned rooting experiment are represented in the figures below.

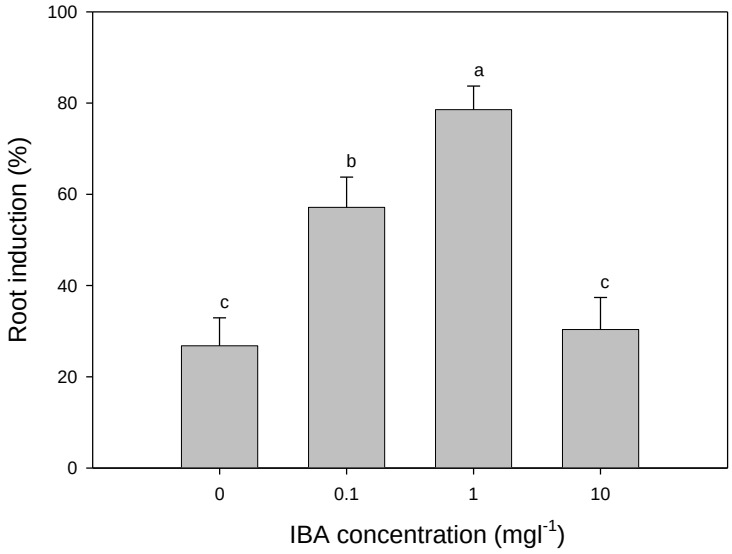


Figure 2.11: The effect of IBA on rooting of bulblets. Bars indicate standard error. Different letters indicate significant differences between treatments at the 5% level (ANOVA).

Figure 2.11 shows the effect that IBA in the basal media has on the percentage of bulblets which rooted on each medium type. The lowest percentage rooting was observed in the control treatment (0 mgl^{-1} IBA), with 27% of bulblets on this medium producing roots. A slightly greater percentage rooting was obtained by bulblets on 10 mgl^{-1} IBA where 30% of bulblets rooted. The differences between these two treatments are not statistically significant. Fifty seven percent of bulblets rooted on the medium supplemented with 0.1 mgl^{-1} IBA. The greatest percentage rooting was obtained on the medium supplemented with 1 mgl^{-1} IBA, with 79% of bulblets producing roots.

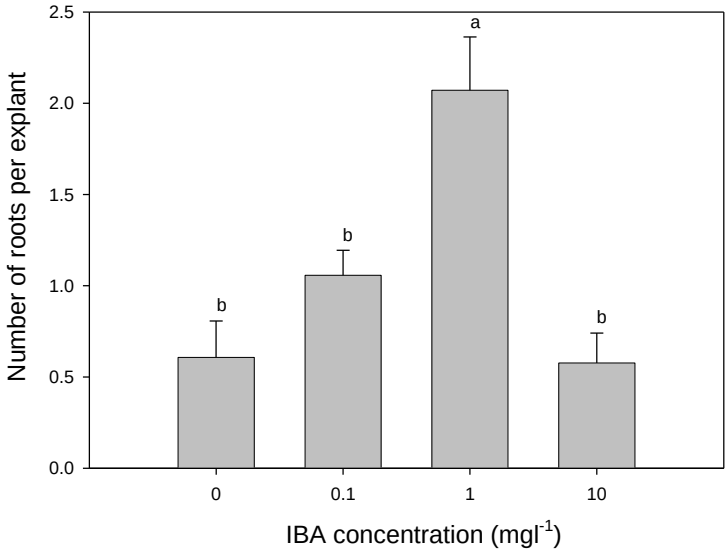


Figure 2.12: The effect of IBA on the number of roots produced by bulblets. Bars indicate standard error. Different letters indicate significant differences between treatments at the 5% level (ANOVA).

Figure 2.12 shows the effect of IBA concentration on the number of roots which the bulblets produced. The fewest roots (an average of 0.6) were formed by bulblets growing on medium supplemented with 10 mgl^{-1} IBA. Bulblets growing on medium without IBA produced on average 0.63 roots per bulblet. Bulblets growing on medium supplemented with 0.1 mgl^{-1} IBA formed 1.1 roots per bulblet. The differences between these three treatments were not significant. The greatest number of roots per bulblet (2.1) were obtained from those bulblets growing on medium supplemented with 1 mgl^{-1} IBA.

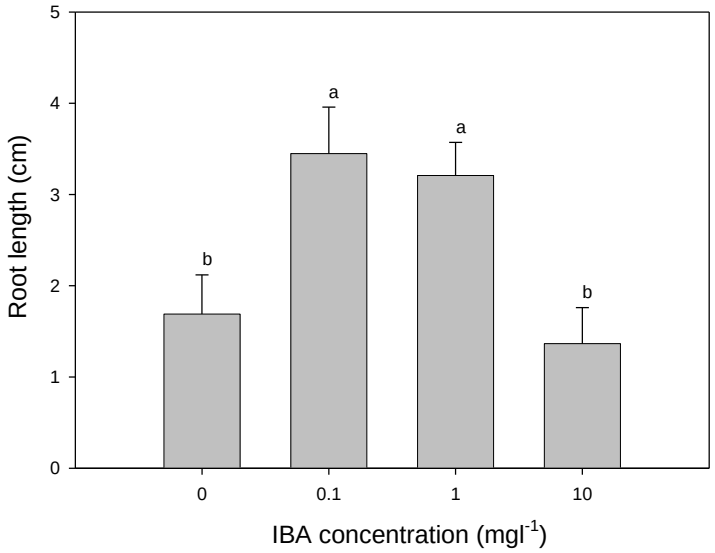


Figure 2.13: The effect of IBA on the length of roots produced by bulblets. Bars indicate standard error. Different letters indicate significant differences between treatments at the 5% level (ANOVA).

Figure 2.13 shows the effect which IBA concentration in growth media has on the length of roots which are produced by bulblets growing on the media. The shortest roots (1.4 cm) were produced by the bulblets growing on medium supplemented with 10 mgl^{-1}IBA. The roots produced on this medium were shorter than roots formed by bulblets growing on the control medium (1.7 cm). However, this difference was not significant. Bulblets growing on medium supplemented with 1 mgl^{-1} IBA were on average 3.2 cm long. The longest roots were formed by bulblets growing on medium supplemented with 0.1 mgl^{-1} IBA. Bulblets on this medium formed roots with an average length of 3.5 cm.

A number of shoots cultured on the rooting media produced shoot clumps. The results of this multiplication are shown in Figure 2.14.

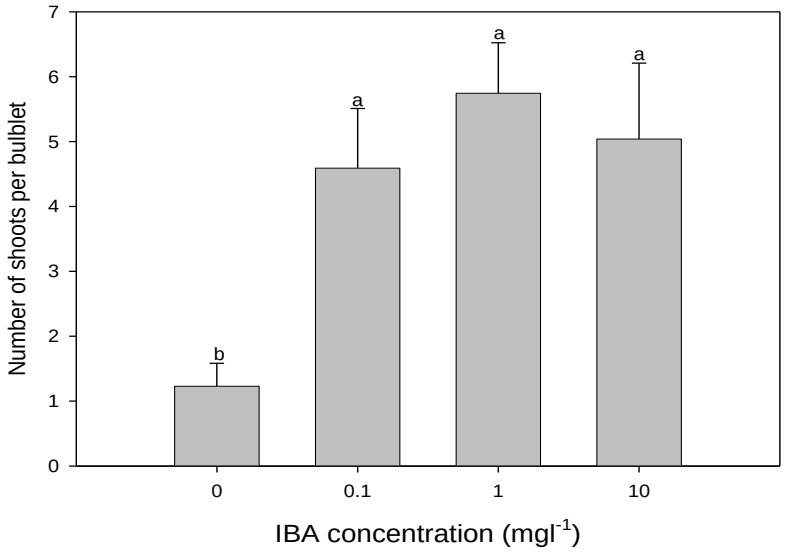


Figure 2.14: The effect of IBA concentration on the number of shoots produced by shoot clusters formed by bulblets cultured on media supplemented with different concentrations of IBA. Bars indicate standard error. Different letters indicate significant differences between treatments at the 5% level (ANOVA).

Figure 2.14 shows the effect that different concentrations of IBA, in a tissue culture medium, had on the number of shoots produced in shoot clusters formed by individual shoots cultured on these media. The inclusion of IBA in the growth medium significantly enhanced the number of shoots formed by bulblets when compared to the control treatment. The number of shoots formed increased as the concentration of IBA increased up to 1 mgl^{-1} IBA.

Callus sub-cultured on MS without hormones produced further shoot clusters (Figure 2.15). An average of 5.3± 0.9 shoots were produced by each explant.

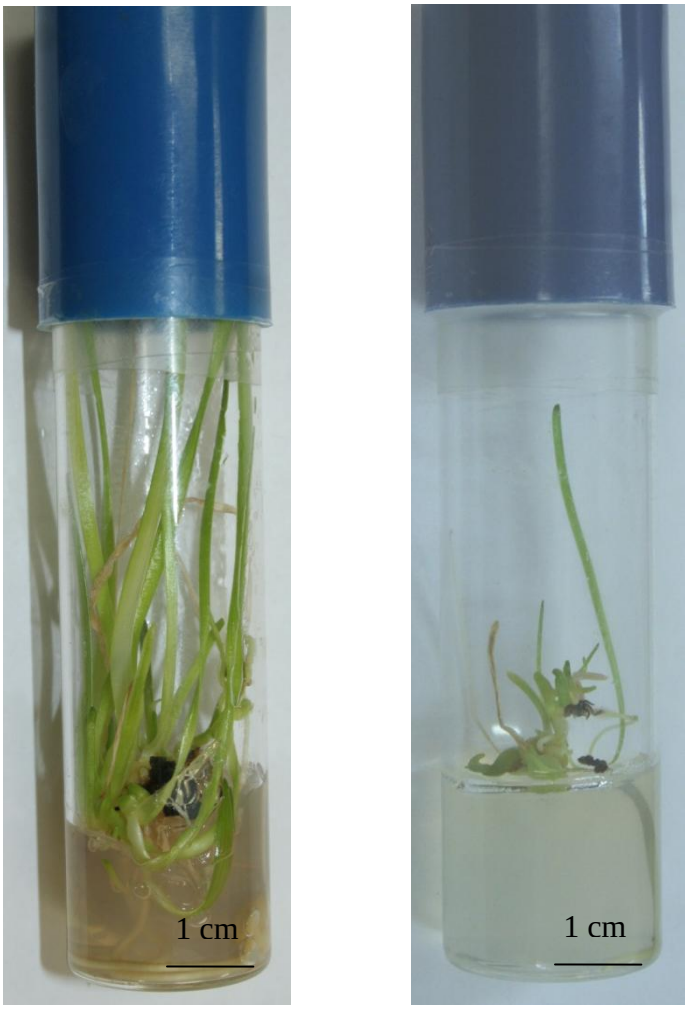


Figure 2.15: Shoot clusters produced by callus placed back onto hormone-free MS medium.

Discussion

The percentage of bulblets which rooted increased with an increase in IBA concentration up to 1 mgl^{-1}. The greatest percentage rooting was obtained with bulblets growing on medium supplemented with 1 mgl^{-1} IBA. The optimum IBA concentration for root induction of *Scilla siberica* bulblets was 0.1 mgl^{-1} (CHAUDHURI & SEN, 2002). The inclusion of IBA in the growth medium at a concentration of 10 mgl^{-1} decreased the percentage of bulblets which formed roots. The percentage root production in this treatment was still higher than the percentage root production in the control treatment. Therefore IBA promoted root induction of bulblets of *Brunsvigia undulata*.

The number of roots produced by each bulblet increased as the concentration of IBA increased up to 1 mgl^{-1}. These results are comparable to similar work done on other bulbous species. The number of roots formed per bulblet of *Eucharis grandiflora* was increased by IBA (PIERIK *et al*., 1983). The number of roots formed by *Lilium lonfiglorum* pseudo-bulblets was enhanced by the inclusion of auxin in the media compared with the control (NHUT, 1998). The inclusion of 10 mgl^{-1} IBA in the medium decreased the number of roots produced per bulblet in comparison to the control. The number of roots produced by bulblets of *Brunsvigia undulata* was increased by IBA at 1 mgl^{-1}.

The length of roots produced by bulblets increased with the inclusion of IBA at a concentration of 0.1 mgl^{-1} in the growth medium when compared with the control. However, the length of roots produced decreased as the concentration of IBA increased above 0.1 mgl^{-1}. IBA at higher concentrations suppresses root growth, resulting in shorter roots in these treatments.

The medium supplemented with 1 mgl^{-1} IBA attained the highest root induction and the greatest number of roots per bulblet. The longest roots were produced by bulblets growing on the medium supplemented with 0.1 mgl^{-1} IBA. However, the difference in the length of roots produced in this treatment and those produced by bulblets on the medium supplemented with 1 mgl^{-1} IBA is marginal, only 0.3 cm. In conclusion, 1 mgl^{-1} IBA is the optimum IBA concentration for rooting of bulblets of *Brunsvigia undulata*.

Unexpectedly shoot clusters were produced by some of the shoots placed on media supplemented with IBA. The inclusion of IBA in the media greatly improved shoot production by bulblets when compared to the mean (Figure 2.14). These results are in agreement with the results of ANGULO *et al*. (2003) who found that high concentrations of auxin enhanced shoot formation in *Cyrtanthus* species.

Sub-cultured callus did not produce shoots as readily as it did before. This may be due to aging of the callus. The induction of shoots of *Scilla siberica* from callus was dependant on the age of the callus (CHAUDHURI & SEN, 2002).

The formation of bulblets *in vitro* eliminates the need for a rooting stage (FENNELL *et al*., 2001). Shoots formed bulblets on rooting media which were then removed from culture and acclimatized according to the method outlined in Section 3.3 on page 115.

2.6 MAINTENANCE OF CALLUS CULTURES

Introduction

Callus can continue to regenerate shoots if sub-cultured at regular intervals, allowing for callus stocks to be maintained for years. These stocks can then be used to induce shoot or bulblet formation. There are disadvantages to using callus to multiply propagules. One of these is that callus loses its regeneration potential.

Materials and methods

Shoot clumps formed from callus cultures were once again separated out after 8 weeks in culture. Individual shoots were placed on rooting media, to prepare them for transfer to the natural environment. The callus left after shoot clusters had been separated was dissected into 5 x 5 mm explants and placed onto the sterilized MS. The culture conditions were as stated in the Materials and Methods on page 22.

Results

Callus formed shoot clusters producing on average 4.4 ± 1.7 shoots per cluster formed from a single callus explant.

Discussion

As observed previously, although callus produced shoots, it was not on as prolific a scale as previously. The first sub-culture of callus explants formed 5.3 shoots in a shoot cluster while the second sub-culture of callus only formed 4.4 shoots per shoot cluster. Age has an effect on the regeneration potential of callus explants (CHAUDHURI & SEN, 2002). Callus loses its regeneration ability over time (HUSSEY, 1975a) and it can stop producing totipotent cells (KIM & DE HERTOGH, 1997). The older the callus the fewer shoots produced. This explains the decrease in the number of shoots formed by callus in the second sub-culture compared with the first sub-culture. The regeneration potential of this callus will continue to decrease. It will not be able to be maintained indefinitely. If callus is to be used for large scale production of shoots, callus stocks should be continuously reintroduced into the system.

CONCLUSIONS

Using seed as explants is not destructive to the plant populations. Many protocols for the tissue culture of bulbous species use bulb explants. This means that the whole plant is destroyed to obtain explants. The collection of seed does not damage the plant in any way and the bulb is left to grow and produce further seed. If some seed is left to germinate in the field, then seed collection will not have a detrimental effect on the wild plant population.

The protocol for the tissue culture of *Brunsvigia undulata* presented above is relatively easy and cost effective. Tissue culture is an expensive technique. This protocol does not require hormones to obtain satisfactory regeneration of bulblets. Only low concentrations of IBA are required to enhance rooting of bulblets. The procedure is not labour intensive either. Seeds germinate easily and they are easy to decontaminate. Many bulblets may be produced using the above protocol.

One seedling can be dissected into ten sections. Three of these sections will form bulblets, shoots and callus. If, for example each of these three sections formed only one bulblet and one shoot and only enough callus to obtain three subcultures, then the bulblet and the shoot would grow and root resulting in two plantlets from each of the three sections - six plantlets. Each of the nine sub-cultured callus explants would form an average of 10.1 shoots each. These 91 shoots would, when sub-cultured, form bulblets and roots. This gives 97 plantlets from one seed. The callus remaining after shoot clusters could be sub-cultured once again. Each of these cultures would form an average of 5.3 shoots each, which could from bulblets with roots. This adds a further 48 plantlets to the 97 plantlets already obtained. With this protocol a conservative average of 145 plantlets may be obtained from one seed.

Chapter 3

TISSUE CULTURE FROM VEGETATIVE EXPLANTS

3.1 TISSUE CULTURE FROM TWIN-SCALES

INTRODUCTION

Twin-scale explants comprise of two adjacent scales connected by a piece of basal plate tissue. Twin-scales have been successfully used as explants in the establishment of cultures in many bulbous species (Table 1.1). Bulbous species successfully produce adventitious shoots from tissue at the base of bulb scales (ROBB, 1957; HUSSEY, 1986), and from the junction of twin-scales on the basal plate (FENNELL, 2002; HAN *et al*., 2005). Twin-scales can produce bulblets directly (HUANG *et al*., 1990a).

MATERIALS AND METHODS

An overview of the Materials and Methods used to generate and multiply bulblets from twin-scales is presented in Figure 3.1 (see p. 56). The outer scales were peeled away from the bulb. Leaves and roots were excised from the bulb. Bulbs were dissected longitudinally into segments and twin-scales were excised from bulb segments (A). Twin-scales were placed in culture. The effects of hormones, activated charcoal, photoperiod, explant origin from within the parent bulb and explant orientation were investigated (B). Bulblets shoots and callus were formed on twin-scales (C). Callus was sub-cultured with no success. Whole bulblets and half-bulblets were sub-cultured onto medium supplemented with 0 mgl^{-1}, 0.1 mgl^{-1}, 1 mgl^{-1} and 10 mgl^{-1} BA to induce multiplication (D). The effects of photoperiod, activated charcoal, sucrose concentration, temperature and liquid culture on bulblet multiplication were investigated (E). Multiplication experiments showed limited success (F). Bulblets which were formed

during multiplication experiments were planted out in a 1:1 vermiculite: perlite potting mixture and placed in a mist house to acclimatize (G).

Unless otherwise stated the growth medium used was MS (MURASHIGE & SKOOG, 1962) with 0.1 gl^{-1} *myo*-inositol, 8 gl^{-1} agar (Agar Bacteriological-Agar No. 1, Oxoid Ltd., England) and 3% sucrose. The culture medium was sterilized by autoclaving for 20 min at 121 °C and 103.4 kPa.

Explants were placed on 10 ml of medium in 40 ml culture tubes which were sealed with metal caps and a 1 cm thick strip of Parafilm®. Cultures were kept at 25 ± 1 °C under Osram® 75 W cool white fluorescent tubes in a 16 h photoperiod with a light intensity of 74.4 µmol m^{-2} s^{-1}. Unless otherwise stated 25 twin-scales were used per treatment.

Where results were recorded as percentages, the data were arcsine transformed prior to statistical evaluation (SCOTT *et al.*, 1984). An analysis of variance (ANOVA) was carried out on all data. Data were analysed using a Duncan's test at the 5% level in SPSS (Statistical Package for the Social Sciences), version 10.0.

3.1.1 Plant collection and decontamination

Introduction

Contamination is the largest problem faced when establishing cultures from soil-born explants such as bulb explants (FENNELL & VAN STADEN, 2004). Bulbs are in direct contact with the soil which makes them very difficult to decontaminate (HUSSEY, 1975b). Contamination rates are often high in plant tissue cultures which have been initiated from bulbs, rhizomes and stolons (HOL & VAN DER LINDE, 1992). Bacteria, fungi, other micro-organisms, mites and thrips which are found in the soil can contaminate cultures (HOL & VAN DER LINDE, 1992). For this reason the use of a strong sterilant is necessary when preparing bulbous explants for tissue culture.

Figure 3.1 A-C

Figure 3.1 D-G

The use of 70-90% ethanol as a pre-treatment is often used (WITOMSKA & LUKASZEWSKA, 1997) as this allows for better penetration of the decontaminating agent (DODDS, 1991). While bulbous plant material requires a fairly harsh decontamination regime, the regeneration potential of the explant may be affected by damage caused and cell or tissue death. The strength of the decontaminating agent as well as the time for which the plant material is soaked is important.

Materials and Methods

Brunsvigia undulata F.M. Leight. plants were collected form Mount Gilboa forestry estate (29° 16.764′ S, 30° 17.627′ E) in early February 2007. Whole plants were dug up after flowering and seed dispersal, but before leaf senescence. The bulbs collected were approximately 10 cm in diameter. Three bulbs were used for the decontamination experiment.

All leaves were removed from the plant. The bulbs were washed in tap water to remove any sand or soil. The roots and a thin layer of the basal plate were removed and the top two thirds of the bulbs was cut away. The brown outer most scales were peeled away from the bulbs to expose their white inner scales.

Peeled and trimmed bulbs were treated with 1% Benlate® for 15 min after which the Benlate® was washed from the bulb with sterile distilled water. Bulbs were then decontaminated using one of two decontamination procedures:

1. Bulbs subjected to the first decontamination procedure were dipped, whole, in 70% ethanol for 1 min and then in 0.2% mercuric chloride with a few drops of Tween 20 for 10 min. Bulbs were rinsed with sterile distilled water and then dissected in half longitudinally. Half-bulbs were put into ¾ strength Jik (2.6% sodium hypochlorite) with a few drops of Tween 20 for 10 min. Thereafter half-bulbs were rinsed with three changes of sterile distilled water.

2. The second decontamination procedure involved bulbs being decontaminated for 15 min in 0.2% mercuric chloride with a few drops of Tween 20. Bulbs were not dipped in 70% ethanol. Bulbs were subsequently rinsed with sterile distilled water to remove the sterilant. Bulbs were dissected in half and soaked in 0.1% mercuric chloride with a few drops of Tween 20 for 10 min after which they were washed with three changes of sterile distilled water.

Results

Explants from bulbs which were subjected to decontamination procedure 1 were dehydrated and turned brown. The explants from bulbs subjected to decontamination procedure 2 remained white or turned green.

Table 3.1 shows the percentage of cultures which were alive and not contaminated after decontamination and 14 days in culture. Decontamination procedure 2 was more successful than decontamination procedure 1. The first decontamination procedure resulted in very poor explant survival. Only 5% of these cultures remained free of contaminants and alive. Greater success was obtained with decontamination procedure 2 where 69.8% of cultures survived and were not contaminated.

Table 3.1: The percentage of cultures which remained free of contamination after undergoing different decontamination procedures.

Procedure	Survival and decontamination (%)
1	5
2	69.8

Discussion

The open structure of bulbs allow for micro-organisms to penetrate deep into the bulb moving between the bulb scales. This makes bulbs very difficult to free from contaminants (HUSSEY, 1975b). High contamination rates are often the cause for failed micropropagation using bulbous explants.

Twin-scales which were subjected to decontamination procedure 1 were discarded due to contamination and death. Although not all these twin-scales were contaminated, those which were dehydrated would not have been productive in culture. The use of alcohol in a decontamination procedure is to remove waxes from the surface of the plant material, allowing sterilants to penetrate the tissue (GEORGE, 1993). The 70% ethanol step in the first decontamination procedure caused the twin-scales to dehydrate. The bulb scales of *Brunsvigia undulata* are very thin and 1 min in 70% ethanol proved to be a detrimental step in decontamination. Treatment with ethanol for a shorter time may have been more successful and beneficial to the decontamination procedure.

The second decontamination procedure was more successful. After being subjected to this decontamination procedure 69.8% of twin-scale explants were not contaminated. The exclusion of an ethanol step from this procedure prevented dehydration of twin-scales. In bulbous species contamination is a "major problem" and often results in a "significant loss of cultures" (KIM & DE HERTOGH, 1997). WITOMSKA and LUKASZEWSKA (1997) observed contamination rates of between 70 and 90% for scale segments from *Fritillaria imperialis*. Decontamination rates of 60% were the highest achieved by HUSSEY (1975b) when testing different decontamination methods for *Hyacinthus* bulb explants.

Mercuric chloride is a more aggressive sterilant than sodium hypochlorite and is effective in decontaminating bulbs (FENNELL, 2002). Heavy metal ions that are toxic to plant tissues are present in mercuric chloride (FENNELL, 2002), restricting its use to low concentrations only. A concentration of 0.2% is a relatively high concentration of

mercuric chloride for use in decontamination. This concentration ensured that contaminants were removed from the bulbs.

Bulb tissue is extremely difficult to decontaminate and so 69.8% decontamination was considered successful enough to warrant the use of the second decontamination procedure for use in further twin-scale tissue culture experiments.

3.1.2 Establishment of cultures

Introduction

Twin-scales were first used for *Narcissus* (BRUNT, 1985), and are now widely used for the micropropagation of members of the Amaryllidaceae, Liliaceae, Hyacinthaceae and Iridaceae. Twin-scales excised from bulbs comprise of two adjacent scales connected by a piece of basal plate tissue. It is necessary to include the basal plate tissue in the explant (GEORGE, 1993) as in the Amaryllidaceae no bulblets formed without the presence of basal plate tissue (FENNELL, 2002; FENNELL & VAN STADEN, 2004). When the basal plate is cut, apical dominance is alleviated and the out growth of pre-existing axillary meristems is stimulated (FENNELL *et al*., 2001).

The regeneration potential of bulb explants is species and genotype dependant and the number of shoots or bulblets per explant can range from 2 to as many as 25 (ZIV & LILIEN-KIPNIS, 2000).

Materials and Methods

Decontaminated bulbs were dissected longitudinally into six segments. Twin scales joined by 5 mm of the basal plate were excised from these segments. Twin scales were placed adaxial side down onto sterilized MS medium. The culture conditions were as stated in the Materials and Methods on page 55.

Results

Bulblets formed on twin-scales from the basal plate, either from between the scales (Figure 3.2 A) or outside of the twin-scales (Figure 3.2 B). All bulblets formed from the basal plate of the twin-scale. Bulblets formed were made up of between 3 and 5 bulb scales each. There were two ways in which bulblets formed. Either shoots were produced which then developed a bulblet at their base. Alternatively bulblets formed from the base upwards. The scales of the bulblet would grow forming a rosette like structure. The scales would then continue to grow to form a complete bulblet.

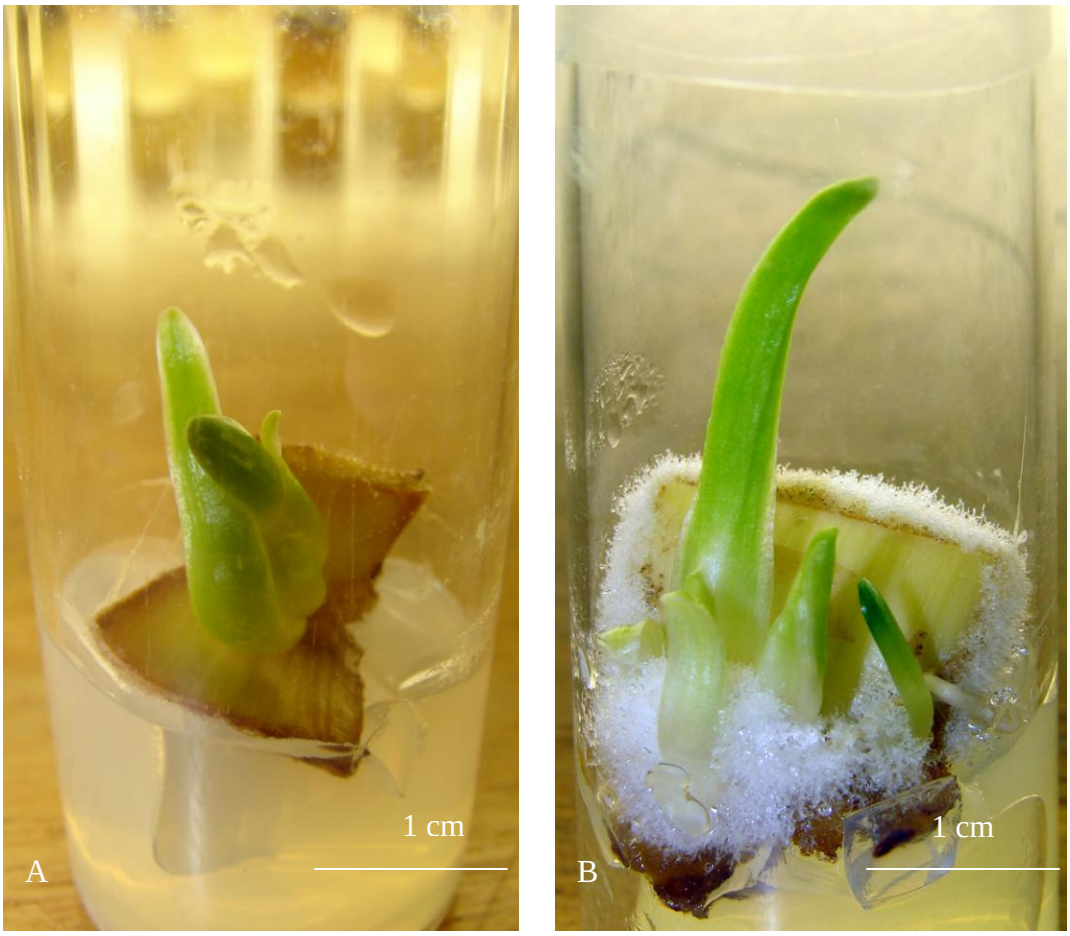


Figure 3.2: Bulblets which formed from the basal plate of twin-scale explants on hormone-free MS medium. Bulblets formed between the scales (A) or on the outside of the twin-scale (B).

Discussion

Twin-scales are superior to single scales for bulblet production (HUANG *et al*., 1990a; HUANG *et al*., 1990b). This is due to the inclusion of a region of basal plate tissue in twin-scales (HUSSEY, 1982; STEINITZ & YAHEL, 1982; JHA *et al*., 1984). Bulblets form between the two scales and near the basal plate, and in some cases bulblets formed on the outside of the twin-scale (DE BRUYN *et al*., 1992). Bulblets formed from the basal plate tissue, due to small centres of meristematic cells in the basal plate tissue where the scales join the basal plate (THOMPSON, 1989). These pre-existing axillary meristems (GROOTAARTS *et al*., 1981) will form shoots or bulblets when the bulb is cut (THOMPSON, 1989). When the parent bulb was cut and twin-scales excised, the meristematic cells in the basal plate were induced to proliferate. This accounts for the origin of the bulblets produced by twin-scales of *Brunsvigia undulata* in culture.

3.1.3 The effects of medium constituents in tissue culture

3.1.3.1 The effect of hormone concentration and combinations

Introduction

There are many factors which affect organogenesis *in vitro* with the most important being plant hormones (YEOMAN, 1986). Adventitious shoot meristems may be induced directly on scale explants. This may happen spontaneously, however typically the addition of plant hormones to the medium is necessary (HUSSEY, 1980).

By changing the concentrations of cytokinin and auxin that the explant is exposed to, one can obtain different types of organogenesis (YEOMAN, 1986). High ratios of auxin to cytokinin causes root formation and callus formation in monocotyledonous plants and a low auxin to cytokinin ration results in axillary shoot proliferation (VAN STADEN *et al*., 2008).

The effect of hormones on twin-scales in culture varies depending on the species. Shoot induction from twin-scales is stimulated by the inclusion of plant growth hormones in the medium (FENNELL, 2002). The inclusion of NAA and BA in the growth medium for the tissue culture of some Amaryllidaceae from twin-scales enhanced shoot formation (ANGULO *et al*., 2003). Auxin induced bulblet formation on twin-scales of *Narcissus* (CHOW *et al*., 1992) and inhibited bulblet formation in *Hippeastrum* (MII *et al*., 1974). Cytokinin alone enhanced bulblet formation in *Nerine* (CUSTERS & BERGERVOET, 1992), and inhibited bulblet formation in *Narcissus* (CHOW *et al*., 1992) and *Hippeastrum* (MII *et al*., 1974).

The induction of shoots from twin-scales is regulated by the interaction of auxins and cytokinins (FENNELL, 2002). Bulblet regeneration may be stimulated (TAKAYAMA & MISAWA, 1979) or inhibited (STEINITZ & YAHEL, 1982; MOCHTAK, 1989; DREWES & VAN STADEN, 1994) by a combination of auxin and cytokinin in the medium. High concentrations of auxin and cytokinin inhibited bulblet formation in Tulip (ALDERSON *et al*., 1986), *Hippeastrum hybridum* (MII *et al*., 1974) and *Sternbergia clusiana* (ORAN & FATTASH, 2005) cultures.

Materials and Methods

To determine the effect of hormones on bulblet formation, five concentrations of BA were combined systematically with five concentrations of NAA and included in the medium (Table 3.2). Twin-scales were excised from bulbs as described previously and placed adaxial side down onto the sterilized MS. The culture conditions were as stated in the Materials and Methods on page 55.

Table 3.2: Combinations of BA and NAA used with twin-scale explants.

BA (mgl^{-1}) \ NAA (mgl^{-1})	**0**	**0.5**	**1**	**2**	**10**
0	0:0	0:0.5	0:1	0:2	0:10
0.5	0.5:0	0.5:0.5	0.5:1	0.5:2	0.5:10
1	1:0	1:0.5	1:1	1:2	1:10
2	2:0	2:0.5	2:1	2:2	2:10
10	10:0	10:0.5	10:1	10:2	10:10

Results

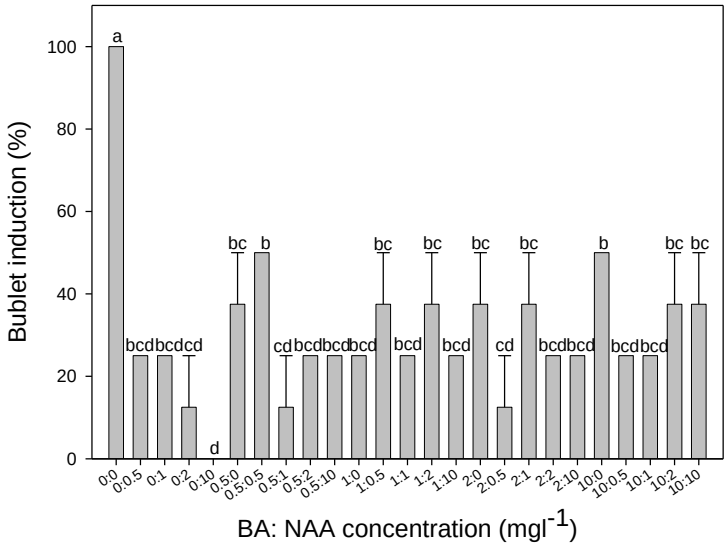


Figure 3.3: The percentage of twin-scale explants which formed bulblets on medium containing different concentrations of BA and NAA. Bars indicate standard error. Different letters indicate significant differences between treatments at the 5% level (ANOVA).

Figure 3.3 shows the percentage of twin-scales which formed bulblets in the presence of different concentrations of NAA and BA. All twin-scales on the control (hormone-free) medium formed bulblets. Half (50%) of the twin-scales on medium supplemented with 0.5 mgl^{-1} BA and 0.5 mgl^{-1} NAA and on medium supplemented with 10 mgl^{-1} BA and

without NAA formed bulblets. Bulblet induction was less than 50% on all other treatments. Twin-scales on medium supplemented with 10 mgl^{-1} NAA and without BA did not form bulblets.

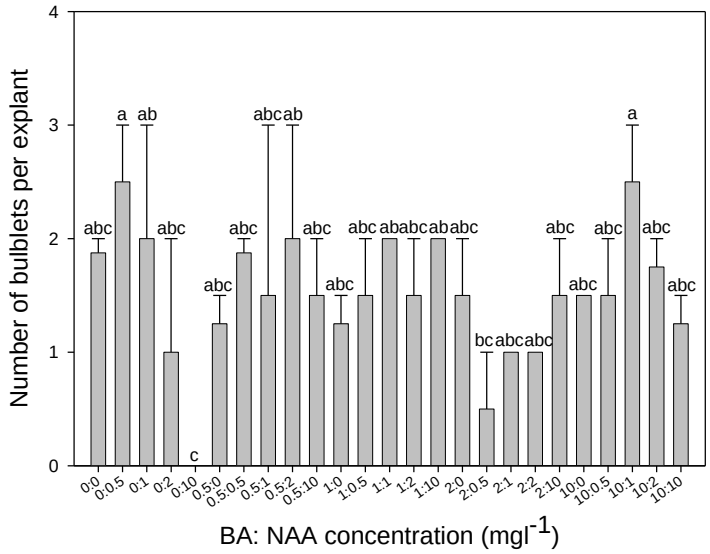


Figure 3.4: The number of bulblets produced per twin-scale on medium supplemented with different concentrations of BA and NAA. Bars indicate standard error. Different letters indicate significant differences between treatments at the 5% level (ANOVA).

Figure 3.4 shows the number of bulblets which were formed by twin-scales on different concentrations of BA and NAA. Twin-scales on medium supplemented with 0.5 mgl^{-1} NAA and without BA and those on medium supplemented with 10 mgl^{-1} BA and 1 mgl^{-1} NAA formed 2.5 bulblets per explant, the greatest number of explants formed per twin-scale in this experiment. At low and intermediate concentrations bulblet production was relatively high when BA and NAA were present in a 1:1 ratio. Bulblets were not formed by twin-scales on medium supplemented with 10 mgl^{-1} NAA without BA.

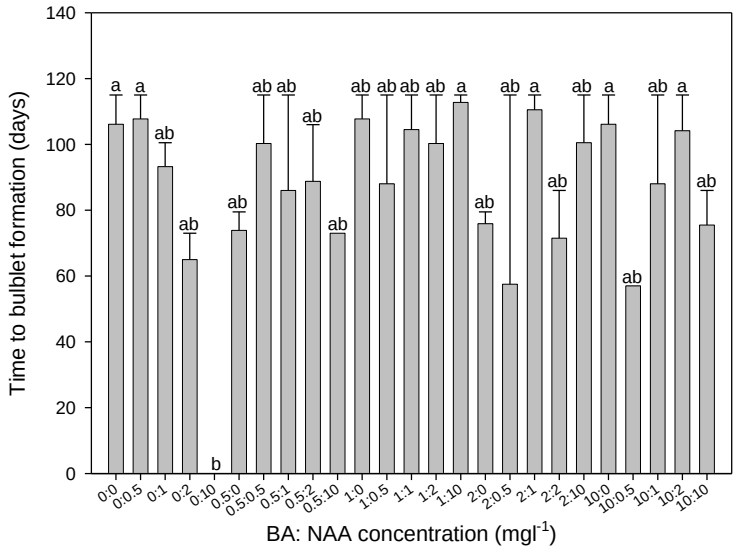


Figure 3.5: The number of days taken for twin-scales to form bulblets on medium supplemented with different concentrations of BA and NAA. Bars indicate standard error. Different letters indicate significant differences between treatments at the 5% level (ANOVA).

Figure 3.5 shows the number of days taken for bulblets to form on twin-scales cultured on different concentrations of BA and NAA. Bulblet formation was quickest in the presence of 2 mgl^{-1} BA and 0.5 mgl^{-1} NAA and 10 mgl^{-1} BA and 0.5 mgl^{-1} NAA. Bulblets formed in 57 days from twin-scales on this treatment.

When BA is absent, present at a very low concentration and at a very high concentration in the medium, an increase in NAA concentration decreased the time taken for bulblets to form.

Bulblets were slowest to form on 1 mgl^{-1} BA and 10 mgl^{-1} NAA, with 112.5 days the average time taken for bulblets to develop. The differences between these treatments were not significantly different.

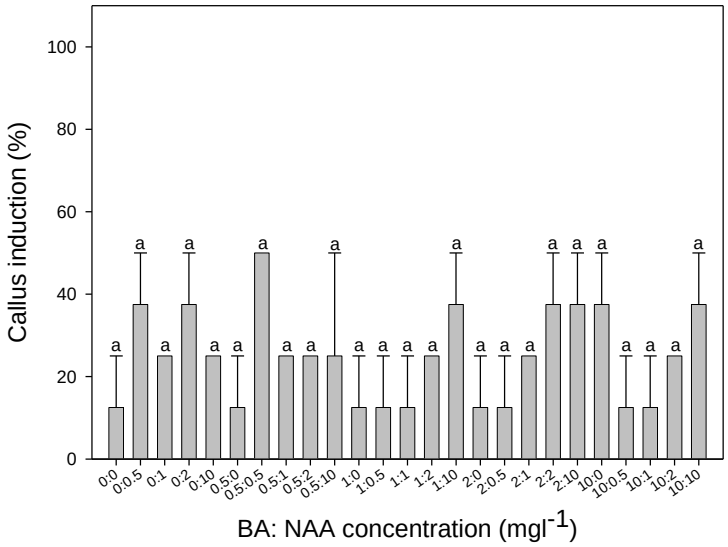


Figure 3.6: The percentage of twin-scales which produced callus on medium supplemented with different concentrations of BA and NAA. Bars indicate standard error. Different letters indicate significant differences between treatments at the 5% level (ANOVA).

The callus formed by twin-scales was very delicate and formed on the cut surfaces of the scales (Figure 3.2 B).

Figure 3.6 shows the percentage of twin-scales which formed callus when placed on different concentrations of BA and NAA. Callus was formed by twin-scales in all treatments. Half (50%) of the twin-scales on medium supplemented with 0.5 mgl^{-1} BA and 0.5 mgl^{-1} NAA formed callus.

Callus induction was relatively high when BA and NAA were present in the medium at a 1:1 ratio at high concentrations and lowest on a hormone-free medium. When BA was present at a very high concentration, in the absence of NAA, callus induction was low.

Discussion

Bulblet induction occurred in all treatments except for one (Figure 3.3). Similarly in *Crinum moorei* bulblets were formed by twin-scales on all hormone treatments (FENNELL, 2002). The highest bulblet induction was seen on the control medium, without hormones. Hormones were not essential for bulblet regeneration from twin-scales. This agrees with results for many other species. Bulblets formed on twin-scales of *Pancratium maritium* with and without hormones in the growth medium (DRAGASSAKI *et al*., 2003), *Narcissus tazetta* (STEINITZ & YAHEL, 1982), *Amaryllis belladonna* (DE BRUYN *et al*., 1992), *Vallota purpurea* (KUKUŁCZANKA & KROMER, 1988), *Crinum macowanii* (SLABBERT *et al*., 1993), *Nerine bowdenii* (MOCHTAK, 1989), *Lilium auratum* (TAKAYAMA & MISAWA, 1979) and *Lilium speciosum* (ROBB, 1957; TAKAMAYA & MISAWA, 1979). The high rate of bulblet induction may be due to the fact that the explant has high enough endogenous concentrations of the hormones to induce bulblet formation (MAESATO *et al*., 1994; FENNELL, 2002). BA and NAA, alone or in combination, decreased the number of twin-scales which formed bulblets compared to the control.

In the absence of BA an increase in NAA concentration had a negative effect on the percentage of explants which formed bulblets (Figure 3.3). Shoot and bulblet formation on twin-scales of *Crinum moorei* (FENNELL, 2002), and *Pancratium maritimum* (DRAGASSAKI *et al*., 2003) was inhibited by the presence of auxin alone. At low concentrations of BA an increase in NAA concentration caused a decrease in bulblet induction.

Low concentrations of BA and NAA (0.5:0.5 mgl^{-1}) showed bulblet induction higher than all other treatments but lower than the control (Figure 3.3). A 1:1 ratio of BA and NAA at 0.5 mgl^{-1} was the optimum hormone treatment for bulblet induction in *Eucomis zambesiaca* (RAMOGOLA & FENNELL, 2007). A fairly high bulblet induction rate was also achieved by a high concentration of BA alone.

Bulblet induction was relatively high when there was a high BA to NAA ratio in the growth medium. A high cytokinin to auxin ratio was found to be successful for bulblet induction from twin-scales of *Narcissus asturiensis* (SANTOS *et al*., 2002). BA increased shoot formation from twin-scales in *Lilium longiflorum* (HAN *et al*., 2004). With high cytokinin concentrations and NAA present, up to 20 bulblets may be produced by *Narcissus* twin-scales (HUSSEY, 1978). Relatively high bulblet induction was observed when the highest concentrations of BA and NAA were used in combination. The greatest number of shoots as well as the greatest percentage of explants producing shoots in *Cyrtanthus* species was achieved with the highest concentrations of both NAA and BA (ANGULO *et al*., 2003).

A high NAA to BA ratio inhibited bulblet production by twin-scales. An increase of NAA greater than 0.01 mgl^{-1} curbed regeneration by twin-scales of *Lilium* (MAESATO *et al*., 1994).

The greatest number of bulblets per explant was obtained by twin-scales on medium with a high NAA to BA ratio and on a medium with a high BA to NAA ratio (Figure 3.4). Both high NAA to BA ratios and high BA to NAA ratios have successfully formed high numbers of bulblets in other Amaryllidaceae species. High NAA to BA ratio increased bulblet production in *Nerine* (PIERIK & IPPEL, 1977). High ratios of auxin to cytokinin (CHOW *et al*., 1992) and high cytokinin to auxin ratios (HUSSEY, 1978) increased bulblet formation in *Narcissus*.

In the absence of BA, low concentrations of NAA increased the number of bulblets formed by twin-scales (Figure 3.4) but did not affect the time taken for bulblets to form (Figure 3.5). It is thought that there are low levels of endogenous cytokinin in twin-scales and so low levels of auxin create a hormone balance which favour organ formation (FENNELL, 2002). However, as NAA concentration increased the number of bulblets produced decreased, but were quicker to form (Figure 3.5). An increasing NAA concentration caused a decrease in organogenesis (DREWES & VAN STADEN, 1994).

In the absence of NAA, BA concentration did not significantly affect the number of bulblets formed per explant. Different levels of hormones did not affect the number of bulblets formed on twin-scales of *Amaryllis belladonna* (DE BRUYN *et al*., 1992). Endogenous level of BA may be sufficient for bulblet formation (MII *et al*., 1974). In this case twin-scales would not use the BA in the medium and so BA concentration would not affect morphogenesis.

When intermediate concentrations of BA are present in the medium, NAA concentration does not affect the number of bulblets formed by twin-scales. This may be as a result of the anti-auxin effect of cytokinins (VAN STADEN *et al*., 2008). Cytokinins can alter the activity of auxins (VAN STADEN *et al*., 2008). This could suppress the impact of auxins on bulblet formation by twin-scales.

Callus formed on twin-scales in all treatments (Figure 3.6) and was not influenced by hormone concentrations. Similarly callus also formed on twin-scales of *Crinum macowanii* on all hormone concentrations used (SLABBERT *et al*., 1993). Callus formed along the cut surfaces of the twin-scales. This callus formation may have been the result of wounding.

3.1.3.2 Use of activated charcoal

Introduction

Activated charcoal is often added to tissue culture medium and has a number of beneficial effects on the culture. Charcoal is made active by treatment with carbon dioxide. This oxidizes the charcoal and gives it adsorbing properties (PAN & VAN STADEN, 1998).

Activated charcoal when added to tissue culture medium promotes the growth of the tissue explant in culture (WEATHERHEAD *et al*, 1978). Activated charcoal has been

used extensively in the induction of bulblets from twin-scales. This is due to its adsorptive properties and its role in darkening the tissue culture medium.

Activated charcoal adsorbs inhibitory substances from the tissue culture medium (WEATHERHEAD *et* al., 1978; HUGHES, 1981; PECK & CUMMING 1986; PAN & VAN STADEN, 1998), inactivating these substances (WANG & HUANG, 1976). This ensures that they do not have an adverse effect on the explant.

When added to growth medium activated charcoal makes the medium black in colour. This darkens the medium and simulates natural soil conditions (WANG & HUANG, 1976). Charcoal reduces browning and the decay of twin scale bases (STEINITZ & YAHEL, 1982) allowing the bases to produce bulblets. It strongly influences bulblet regeneration and size (FENNELL *et al*., 2001) and enhances bulblet regeneration through direct organogenesis (STEINITZ & YAHEL, 1982).

Materials and Methods

To determine the effect of activated charcoal on twin-scales in culture, 5 gl^{-1} activated charcoal was included in the growth medium. The medium was supplemented with combinations of 0, 1 and 2 mgl^{-1} BA and 0, 1 and 2 mgl^{-1} NAA. Twin-scales were excised from bulbs as described earlier. Twin-scales were cultured adaxial side down on the sterilized MS. All other culture conditions were as stated in the Materials and Methods on page 55.

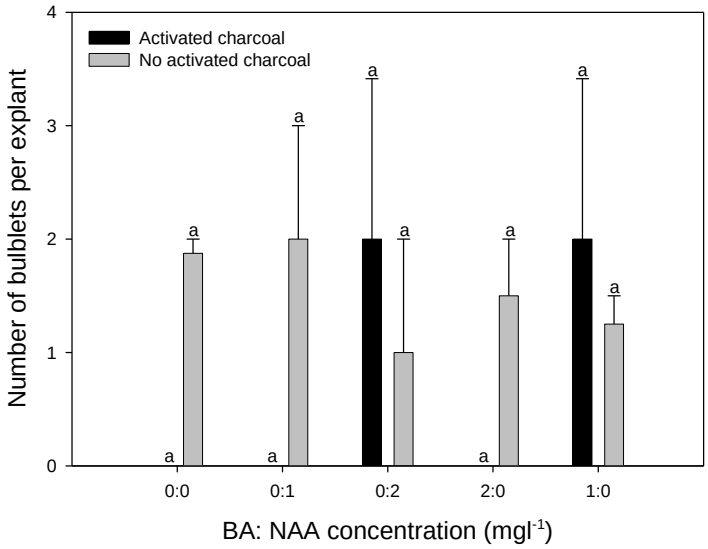


Figure 3.7: The number of bulblets produced by twin-scales cultured on MS medium supplemented with and without activated charcoal and supplemented with different concentrations of BA and NAA. Bars indicate standard error. Different letters indicate significant differences between treatments at the 5% level (ANOVA).

Figure 3.7 shows the number of bulblets which were formed by twin-scales when cultured on medium supplemented with and without activated charcoal. Twin-scales on the activated charcoal control medium, without hormones, on the activated charcoal medium supplemented with 1 mgl^{-1} NAA without BA, and with 2 mgl^{-1} BA without NAA did not produce bulblets. The two activated charcoal treatments which were successful in forming bulblets both formed 2 bulblets per explant. In the absence of activated charcoal, an increase in BA concentration increased the number of bulblets formed. An increase in NAA concentration resulted in a decrease in the number of bulblets produced by explants. The differences between the numbers of bulblets produced by twin-scales were not significant.

Discussion

In the two treatments where bulblets formed in the presence of activated charcoal, activated charcoal increased the number of bulblets produced per explant compared with explants on medium without activated charcoal (Figure 3.7). However, these differences were not significant. Activated charcoal increased the size and number of bulblets formed by twin-scales of *Crinum moorei* (FENNELL, 2002). The inclusion of activated charcoal in the growth medium stimulated bulblet formation in *Crinum moorei* (FENNELL, 2002), *Cyrtanthus* species (MORÁN *et al*., 2003), *Eucrosia* (ZIV & LILIEN-KIPNIS, 2000), *Eucrosia stricklandii* COLQUE *et al*., 2002), *Lilium* (TAKAYAMA & MISAWA, 1980), *Lilium longiflorum* (HAN *et al*., 2004), *Narcissus* (LANGENS-GERRITS & NASHIMOTO, 1997; ZIV & LILIEN-KIPNIS, 2000) and *Nerine* (HAN *et al*., 2005). Enhanced bulblet formation in these two treatments may be due to a darkening of the growth medium, thereby creating conditions which more closely resemble soil conditions (WEATHERHEAD *et al.*, 1978; PAN & VAN STADEN, 1998).

Low concentrations of BA and NAA increased the number of bulblets formed by twin-scales of *Brunsvigia undulata* (Figure 3.4). Activated charcoal has been shown to adsorb auxins, such as NAA and cytokinins, namely BA from tissue culture medium. This renders these hormones inactive (WEATHERHEAD *et al.*, 1978; PAN & VAN STADEN, 1998). The effects of NAA and BA will thus not be exhibited by the explants in culture. This may explain the lack of bulblet formation by twin-scales on medium supplemented with activated charcoal.

3.1.4 Explant factors affecting bulblet formation by twin-scales

Explant polarity and the original position from within the parent bulb may affect the development of bulblets from twin-scales (FENNELL & VAN STADEN, 2004). Explant polarity is an important factor when developing a tissue culture protocol. A change in explant polarity may be the difference between no response in culture and prolific

organogenesis. The scales at different positions in the parent bulb are of different ages, this may affect their response in culture.

3.1.4.1 Explant orientation

Introduction

Twin scale explants may be placed onto the culture medium in a number of different orientations. Explants may react differently depending on which of their surfaces is in contact with the medium. Successful twin-scale culture has been reported for twin-scales placed abaxial side down (LESHEM *et al*., 1982), adaxial side down and upright (CUSTERS & BERGERVOET, 1992; DE BRUYN *et al*., 1992; LANGENS-GERRITS & NASHIMOTO, 1997; COLQUE *et al*., 2002) on the medium. As shoots and bulblets produced by twin scales originate from the basal plate (DE BRUYN *et al*., 1992), it is important that this part of the explant is in contact with the medium. All three of the above mentioned orientations ensure that the basal plate is in contact with the growth medium.

It has been reported that explant orientation affects the success of tissue cultures using other types of explants. Explant orientation is important when placing inflorescence stalk explants of *Narcissus*, *Amaryllis* (HUGHES, 1981), *Nerine* (ALDERSON & RICE, 1986) and *Ornithogalum* (ZIV & LILIEN-KIPNIS, 2000). Changing the orientation of an explant in culture could mean the difference between no regeneration and shoot induction (HUGHES, 1981).

Materials and Methods

Twin scales were placed abaxial side down, adaxial side down and upright on the sterilized hormone-free MS. Twenty five explants were used for each treatment. The culture conditions were as stated in the Materials and Methods on page 55.

Results

The twin scales placed upright and abaxial side down on the medium turned brown and showed no growth response. The adaxial-side-down-scales either remained white or turned green. In addition they produced shoots and bulblets from the basal plate.

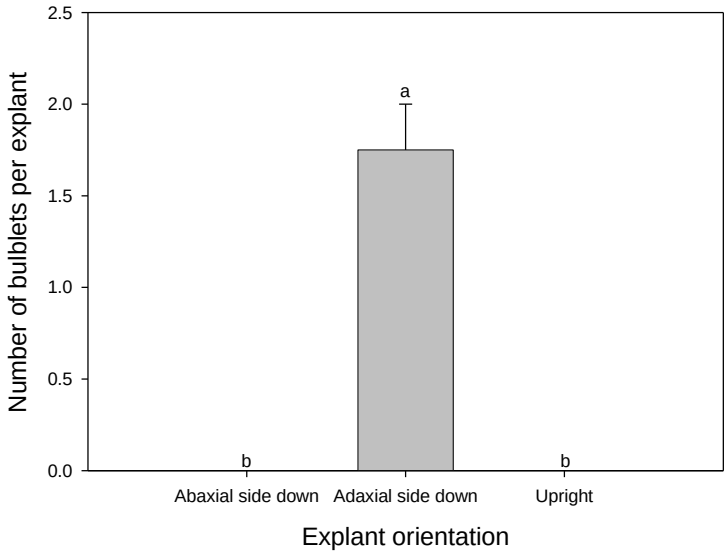


Figure 3.8: The effect of explant orientation on the number of bulblets produced by twin-scales. Bars indicate standard error. Different letters indicate significant differences between treatments at the 5% level (ANOVA).

Figure 3.8 shows the number of bulblets which formed on twin-scale explants placed in different orientations on medium. Bulblets were not produced by twin-scales cultured with their abaxial side in contact with the medium, nor were bulblets produced by twin-scales which were placed upright on the medium with their base embedded in the medium. Twin-scales which were cultured with their adaxial surface in contact with the medium produced an average of 1.7 bulblets per explant.

Discussion

Figure 3.8 clearly shows that explant orientation had an effect on the regeneration potential of twin-scales from *Brunsvigia undulata in vitro*. DE BRUYN *et al*. (1992) found that explant orientation in *Amaryllis belladonna* had no effect on the regeneration potential of the explant.

In the parent bulb a twin-scale is positioned within the bulb so that it is upright. It would follow that twin-scales would be best placed in culture in this upright position. This was not the case when the effect of twin-scale orientation on bulblet formation was investigated. Twin-scales with their adaxial side in contact with the medium were the only ones to form bulblets. It is important, however that the basal plate is sufficiently in contact with the medium. Shoots and bulblets form from the basal plate and so the basal plate must have sufficient access to the nutrients in the medium (DE BRUYN *et al*., 1992). The nutrients and hormones in the medium must be available to the competent tissues (GEORGE, 1993).

Twin-scales placed in culture with their basal plate embedded in the medium did not produce bulblets. Shoots produced by the twin-scales are produced at the basal plate. When twin scales are placed upright in the medium the basal plate is embedded in the medium, restricting oxygen available to the basal plate (PIERIK & RUBING, 1973; FENNELL, 2002). A lack of oxygen will prevent the basal plate from producing shoots or bulblets.

Twin-scales of *Brunsvigia undulata* are best placed in culture with their adaxial side in contact with the medium. This allows for bulblets to form both between the scales and on the abaxial surface of the explant.

3.1.4.2 Explant origin

Introduction

The bulbs of *Brunsvigia undulata* are often quite large and can measure about 15 cm in diameter. The age of the bulbs allow for variation among the bulb scales making up the bulb. Bulb scales of different ages make up different parts of the bulb. New young bulb scales differ from the older bulb scales. It is possible that scales excised from different parts of a bulb would react differently *in vitro*. Scales in different positions in the bulb are different in appearance. Those close to the centre of the bulb are thick and fleshy compared with the paper thin outer bulb scales.

The morphological differences between inner, middle and outer bulb scales suggest that twin-scales from these areas may behave differently in culture. HUANG *et al*. (1990b) found that the thickness of the outer scale of twin-scales used to micropropagate *Hippeastrum hybridum* affected the rate of bulblet formation. Bulblet formation was greatest on explants excised from the outer most scales of the parent bulb (HUANG *et al*., 1990b).

COLQUE *et al.* (2002) investigated the effect that the position of a twin-scale in the parent bulb has on shoot regeneration. HUANG and LIU (1989) excised explants from the inner-most scales. The outer scales are less regenerative than the inner scales (TAKAYAMA & MISAWA, 1980).

Materials and Methods

Twenty five twin scales were excised from three separate areas of two parent bulbs. Between 12 and 15 twin-scales were excised from each position in each bulb. The inner (Position 1), middle (Position 2) and the outer (Position 3) areas. These twin-scales were cultured on sterilized MS. The culture conditions were as stated in the Materials and Methods on page 55.

Results

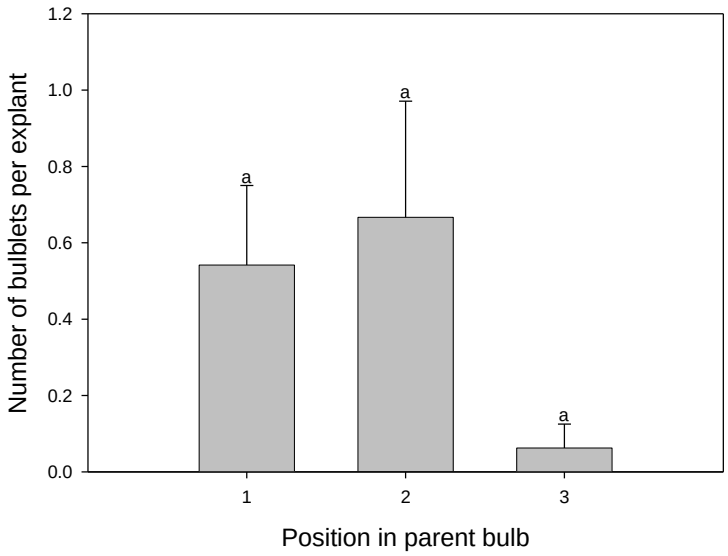


Figure 3.9: The effect of explant position within the parent bulb on the number of bulblets which it produces. Bars indicate standard error. Different letters indicate significant differences between treatments at the 5% level (ANOVA).

Figure 3.9 shows that twin-scales taken from Position 2 in the parent bulb produced the most bulblets per twin-scale. Twin-scales from Position 1 also produced bulblets in culture, however twin-scales from Position 2 produced more bulblets than twin-scales from Position 1. Only one twin-scale from Position 3 produced a single bulblet. The differences between the number of bulblets produced by twin-scales from different positions in the parent bulb were not statistically significantly different.

It was observed that the outer scales were approximately 5 mm thick which is more than double the thickness of the inner scales.

Discussion

The differences in the number of bulblets formed by twin-scales from different positions in the bulb were not statistically significant. Twin scales excised from the inner-most part

(Position 1) of the parent bulb were very thin and fragile. The thinnest twin-scales tended to dry out in culture, hindering their ability to form bulblets. COLQUE *et al.* (2002) found that the inner twin-scales were most productive on most media. When placed on activated charcoal COLQUE *et al.* (2002) found that the outer twin-scales produced the most shoots.

Twin-scales from the middle part (Position 2) of the parent bulb were the most successful in generating bulblets. Twin-scales from this position formed 0.7 bulblets per explant. Twin-scales from this position in the parent bulb also formed the most bulblets in *Crinum moorei* (FENNELL, 2002). The twin-scales from this position are thick and fleshy. It is likely that they have greater food reserves than thinner scales. MYODO and KUBO (1952; cited by TAKAYAMA & MISAWA, 1980) found differences in the levels of soluble nitrogen and sugar between inner and outer scales of *Lilium*. Scales in the middle position of *Narcissus* were highest in both soluble and insoluble sugars (HANKS, 1986). This may explain why bulblet production was highest by twin-scales from Position 2.

A single bulblet was produced by one of the twin-scales taken from the outer part (Position 3) of the parent bulb. This explant may have been the twin-scale which was on the interface between Position 2 and Position 3. COLQUE *et al.* (2002) noted that the outer scales are those which are in direct contact with the sterilant during decontamination. In this experiment a very strong sterilant (mercuric chloride) was used to decontaminate parent bulbs. Mercuric chloride could damage bulb-scales preventing them from forming shoots *in vitro*.

The results of this experiment suggest that the outer part of the parent bulb has little or no regenerative potential *in vitro.* Twin-scales from Positions 1 and 2 are good sources of twin-scale explants for bulblet formation *in vitro*.

3.1.5 Environmental factors affecting tissue culture from twin-scales

3.1.5.1 Photoperiod

Introduction

Photoperiod is an important factor in the success of twin-scale tissue culture. The effect of photoperiod is species specific as twin-scales have been reported to be regenerative under many different light and dark conditions.

Twin-scales of *Eucrosia stricklandii* cultured in darkness induce bulblet formation (COLQUE, *et al*., 2002). *Narcissus* twin-scale explants were most productive when placed in the dark (BERGOÑÓN *et al*., 1996). Bulblet production was reduced by light in *Lilium* and *Narcissus* (STEINITZ &YAHEL, 1982). Light increased the rate of bulblet formation in *Fritillaria* (WITOMSKA & LUKASZEWSKA, 1997), while bulblets only formed in light from twin-scales of *Crinum moorei* (FENNELL, 2002).

In cultures of *Eucharis grandiflora*, no effect on the regeneration of twin-scales was noted when daily exposure to light was extended from 16 h to 24 h (PIERIK *et al*., 1983) and photoperiod had no effect on Tulip bulblet development (ALDERSON *et al*., 1986).

JACOBS *et al*. (1992) found that light increased the number of bulblets formed on twin-scales of *Nerine bowdenii*, while dark increased the number of twin-scales which formed bulblets.

Materials and Methods

The medium was supplemented with 25 different combinations of BA and NAA (Table 3.2). Twin-scales were excised from bulbs as described earlier in this Chapter. Twin scales were placed adaxial side down onto the sterilized MS. Cultures were kept in 24 h

light. All other culture conditions were as stated in the Materials and Methods on page 55.

As a control, twin-scales were placed on the same 25 combinations of hormone as above and kept in a 16 h photoperiod. The culture conditions were as stated in the Materials and Methods on page 55.

Results

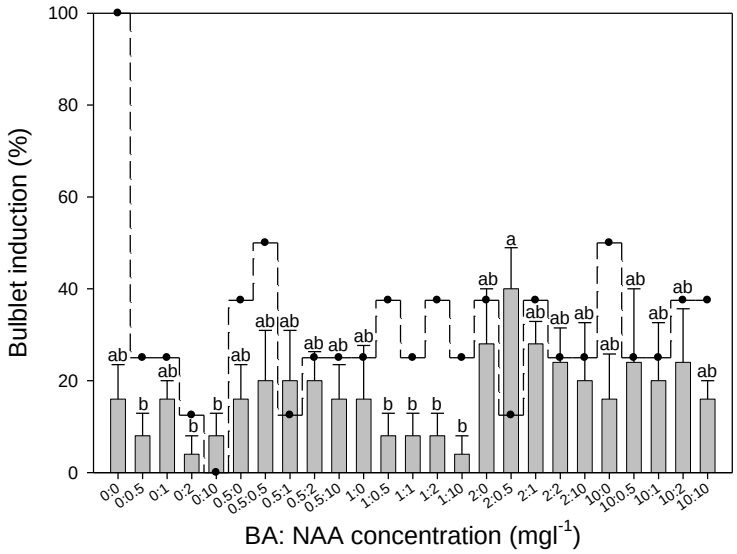


Figure 3.10: The percentage of twin-scale explants which formed bulblets on varying concentrations of BA and NAA when placed under 24 h light conditions. The line indicates the percentage of twin-scales which formed bulblets in a 16 h photoperiod. Bars indicate standard error. Different letters indicate significant differences between treatments at the 5% level (ANOVA).

Figure 3.10 shows the percentage of twin-scales which formed bulblets when placed on different concentrations of BA and NAA and kept under continuous light conditions. For comparative purposes the line on the graph represents the percentage of twin-scales which formed bulblets when placed on the same hormone concentrations but were kept under a 16 h photoperiod. Except for in three treatments, bulblet induction was greater on twin-scales kept under a 16 h photoperiod. The greatest bulblet induction was observed

on twin-scales in continuous light on 2 mgl^{-1} BA and 0.5 mgl^{-1} NAA, where 40% of explants formed bulblets.

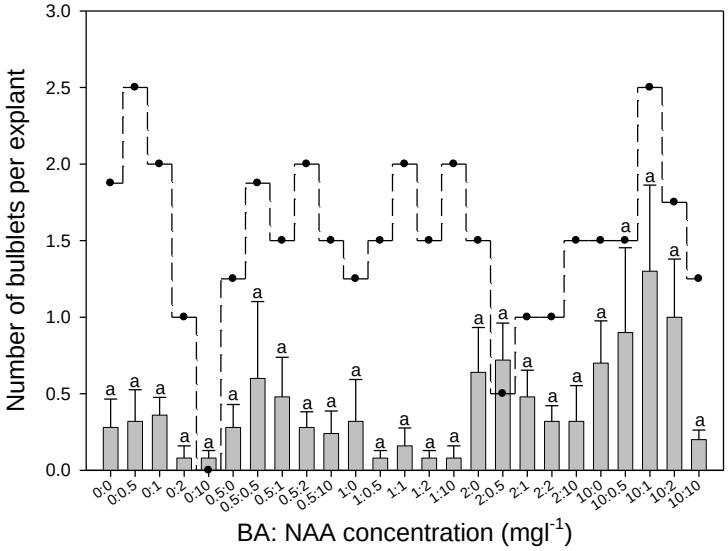


Figure 3.11: The number of bulblets produced by twin-scales on varying concentrations of BA and NAA when placed under 24 h light conditions. The line indicates the number of bulblets produced by twin-scales grown under 16 h photoperiod. Bars indicate standard error. Different letters indicate significant differences between treatments at the 5% level (ANOVA).

Figure 3.11 shows the average number of bulblets formed by twin-scale explants when placed on different concentrations of BA and NAA and kept in continuous light conditions. For comparative purposes the line on the graph represents the number of bulblets formed by twin-scales on the same hormone concentrations but kept under a 16 h photoperiod. Twin-scales grown under continuous light conditions produced fewer bulblets than twin-scales grown under a 16 h photoperiod except in two of the 25 treatments. Twin-scales exposed to light and dark did not produce bulblets when cultured on medium supplemented with 10 mgl^{-1} NAA and without BA. While twin-scales on the same medium, but kept in continuous light, produced 0.08 bulblets per explant. Twin-scales exposed to both light and dark on medium supplemented with 2 mgl^{-1} BA and 0.5 mgl^{-1} NAA produced 0.5 bulblets per explant. Those grown under continuous light on the same medium produced 0.7 bulblets per explant.

An average of 1.3 bulblets per twin-scale was the best bulblet production observed in this experiment. This was achieved on medium supplemented with 10 mgl^{-1} BA and 1 mgl^{-1} NAA. Bulblet induction was greatest when there was a high BA to NAA ratio in the medium. The differences in the number of bulblets produced in the different treatments were not significantly different.

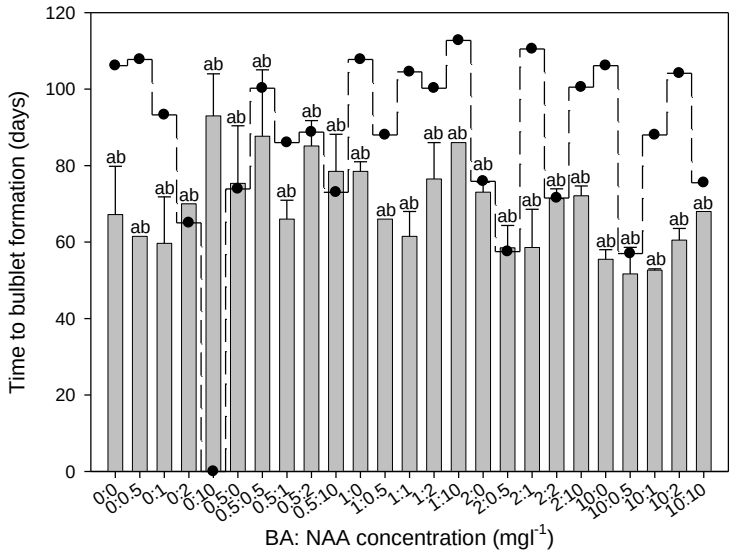


Figure 3.12: The number of days taken for twin-scales to form bulblets on varying concentrations of BA and NAA when placed under 24 h light conditions. The line indicated the number of days taken for twin-scales to form bulblets in a 16 h photoperiod. Bars indicate standard error. Different letters indicate significant differences between treatments at the 5% level (ANOVA).

Figure 3.12 shows the number of days taken for bulblets to form from twin-scales on different hormone concentrations when kept under continuous light conditions. For comparative purposes the line represents the number of days taken for bulblets to form on twin-scales in the same treatments kept in a 16 h photoperiod. The time taken for bulblets to form was the same under both light conditions for twin-scales on medium supplemented with 2 mgl^{-1} BA and 2 mgl^{-1} NAA. Bulblets in this treatment formed within 71.5 days. Bulblets on twin-scales in continuous light took longer to form than those in the 16 h photoperiod when on medium supplemented with 2 mgl^{-1} NAA and without BA and without NAA, 0.5 mgl^{-1} BA and 10 mgl^{-1} NAA and 2 mgl^{-1} BA and 0.5 mgl^{-1} NAA.

In all other treatments, bulblets took fewer days to form on twin-scales in continuous light than those in a 16 h photoperiod. The differences in the time taken for bulblets to form from twin-scales in continuous light are not significantly different.

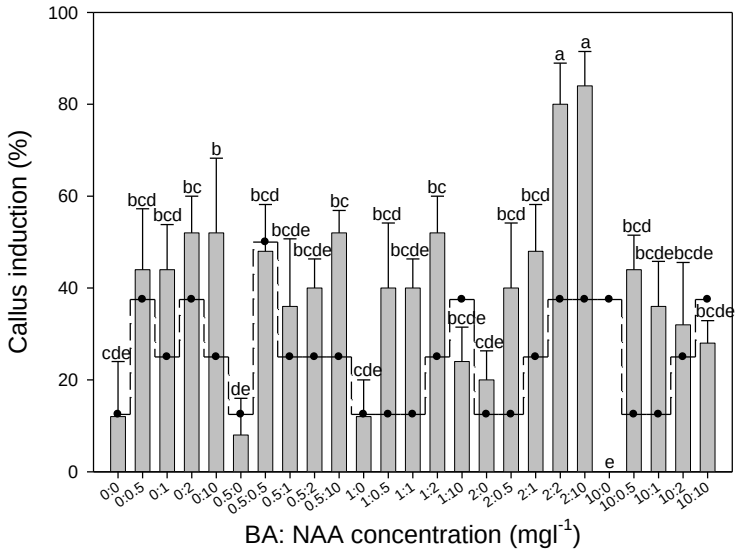


Figure 3.13: The percentage of twin-scales which produced callus on varying concentrations of BA and NAA when placed in a 24 h photoperiod. The line indicates the percentage of twin-scales which produced callus in a 16 h photoperiod. Bars indicate standard error. Different letters indicate significant differences between treatments at the 5% level (ANOVA).

Figure 3.13 shows the percentage of twin-scales which formed callus when placed on growth medium substituted with different hormone concentrations and kept under continuous light conditions. For comparative purposes the line represents the percentage of twin-scales which formed callus on the same treatments but kept under a 16 h photoperiod. In the majority of treatments callus induction was greater in continuous light than it was in a 16 h photoperiod. The percentage of twin-scales forming callus was similar for twin-scales in 16 h light and continuous light, for twin-scales on hormone-free medium and medium supplemented with 1 mgl^{-1} BA and without NAA (12.5% and 12% respectively).

The highest percentage callus induction was observed for twin-scales on medium supplemented with 2 mgl^{-1} BA and 10 mgl^{-1} NAA (84%) and 2 mgl^{-1} BA and 2 mgl^{-1} NAA (82%). The differences between these two treatments were not significantly different. Callus induction was highest when there were high concentrations of both BA and NAA in the medium. In the absence of BA, an increase in NAA resulted in an increase in callus induction. Callus was not formed by twin-scales on medium supplemented with 10 mgl^{-1} BA and without NAA.

Discussion

In general, twin-scales cultured in a 16 h photoperiod had a higher bulblet induction rate than those cultured in continuous light (Figure 3.10), with the exception of two treatments in continuous light. Twin-scales on 10 mgl^{-1} NAA and without BA and those on 2 mgl^{-1} BA and 0.5 mgl^{-1} NAA in continuous light had a higher bulblet induction rate than twin-scales on the same hormones but in a 16 h photoperiod. Bulblet formation is stimulated by a period of darkness in many bulbous species (VAN AARTRIJK & VAN DER LINDE, 1986). Higher rates of regeneration were obtained by exposure to darkness in *Fritillaria imperialis* (WITOMSAKA & LUKASZEWSKA, 1997). A 16 h photoperiod was the optimum for bulblet formation by *Fritillaria thunbergii* (PAEK & MURTHY, 2002).

Twin-scales under 16 h light and 8 h dark produced more bulblets per explant than twin-scales, on the same medium, under continuous light (Figure 3.11). A greater number of bulblets were formed on twin-scales of *Cyrtanthus* species (MORÁN *et al*., 2003) and *Lilium* species (VARSHNEY *et al*., 2000) in a 16 h photoperiod. Bulblet formation was greater in *Nerine* when twin-scales were exposed to a period of darkness (PIERIK & IPPEL, 1977). This was seen in all but two treatments. The differences, however, between the number of bulblets formed in these two treatments in different photoperiods is very small.

These results suggest that twin-scales require exposure to a dark period for increased bulblet induction and production. Twin-scale explants are derived from bulbs. As bulbs are soil-bound they are exposed to little if any light in their natural habitat. Dark conditions simulate the underground conditions in which bulbs normally grow (ULRICH *et al*., 1999).

In most treatments, bulblets took fewer days to form in continuous light than those in a 16 h photoperiod (Figure 3.12). Bulblets are green indicating the presence of chlorophyll and the ability to photosynthesis. The exposure to a greater number of hours of light will enable greater photosynthesis by the explants in continuous light, compared with those in a 16 h photoperiod. *Hippeastrum* is cultured in continuous light (HUANG *et al*., 1990a). Greater photosynthesis will mean greater production of carbohydrates and so the potential for increased growth and regeneration. Growth and regeneration would take place faster under these conditions. The hormone-directed transport of assimilates may be affected by light (LESHEM *et al*., 1982). This would also contribute to an increased growth rate under continuous light conditions.

The induction of callus on twin-scales was greater under continuous light than in a 16 h photoperiod (Figure 3.13). This may be attributed to greater photosynthesis under continuous light. This will lead to the explant having greater energy available for callus formation.

3.1.6 Sub-culture of callus from twin-scales

Introduction

Callus can be a very successful way of producing organs in culture. Indirect morphogenesis occurs when callus is induced to produce plant parts, such as roots, shoots or even bulblets and corms. “Callus cultures range from friable callus consisting of parenchyma-type cells to highly organized masses with some properties of organ

primordia" (YEOMAN, 1986). This is determined by the type of plant tissue and the plant species from which it had come (YEOMAN, 1986).

Materials and Methods

The medium was supplemented with 5 mgl^{-1} BA: 0 mgl^{-1} NAA, 5 mgl^{-1} BA: 0.1 mgl^{-1} NAA, 5 mgl^{-1} BA: 1 mgl^{-1} NAA, and 5 mgl^{-1} BA: 5 mgl^{-1} NAA.

Callus produced by twin scales in culture was removed and placed on the sterilized MS. Half of the cultures were placed under Osram® 75 W cool white fluorescent tubes in a 16 h photoperiod with a light intensity of 34.2 µmol $m^{-2}s^{-1}$. The other half of the cultures were placed in continuous dark conditions. Fifteen callus explants were cultured per treatment. All other culture conditions were as stated in the Materials and Methods on page 55.

Results

Neither callus cultures in the 16 h photoperiod nor those in the dark showed any growth or morphogenesis. Within a week of being sub-cultured, callus cultures turned brown.

Discussion

Callus from other Amaryllidaceae were also not very responsive in culture. Callus explants of *Crinum moorei* grew very slowly when sub-cultured (FENNELL, 2002). It is possible that an increase in phenolics in the medium inhibited callus growth. There may have been an increase in the production of phenolics (GEORGE, 1993), which would have been toxic to the explants and prevented them from growing. This may explain the lack of growth response by the callus explants. In conclusion, callus formed by twin-scales was not viable for sub-culture.

3.1.7 Multiplication of bulblets

Shoots which grow from *in vitro* formed bulblets are initiated by multicellular meristems (HUSSEY, 1982). This makes bulblets a good source of explants for *in vitro* multiplication of bulbous species. The propagules will be genetically uniform (HUSSEY, 1982).

Storage organ formation is affected by sucrose concentration, the balance of plant growth regulators, charcoal, temperature and occasionally, the photoperiod (FENNELL & VAN STADEN, 2004). Bulblets formed *in vitro* often become dormant when sub-cultured. The removal of the leaves and upper part of the bulblets will help to prevent dormancy (CUSTERS & BERGERVOET, 1992). When not in a state of dormancy *in vitro* grown bulblets are a successful explant for bulblet multiplication (DE BRUYN *et al*., 1992).

It is common for bulblets to enter into a state of dormancy. Cutting the bulblet will break dormancy and induce shooting (FENNELL, 2002). Removing the shoots and the top of the bulblet removes the shoot apex. This will affect axillary shooting (FENNELL, 2002).

Splitting bulblets in half will also have an effect on shooting and multiplication. Apical dominance in *Narcissus* is exhibited by the basal plate (CHOW *et al*., 1992). Cutting the basal plate will therefore break apical dominance.

3.1.7.1 The effect of cytokinin concentration on multiplication

Introduction

Cytokinins are known to release axillary meristems from apical dominance. Therefore their inclusion in the growth medium for the proliferation of shoots and bulblets from *in vitro* formed bulblets, may initiate the growth of inhibited lateral buds (HUSSEY, 1986). The axillary shoots released from apical dominance may in turn produce their own precocious axillary shoots and so on. As the axillary meristems in bulblets are in the basal

plate (DE HERTOGH & LE NARD, 1993; FENNELL, 2002), cytokinins in the medium will be easily accessible for use by the basal plate of the bulblet (HUSSEY, 1976).

BA causes branching of shoots in bulbous species (HUSSEY, 1976). FENNELL (2002) found that divided bulblets of *Crinum moorei* placed on medium containing BA enhanced shoot initiation. An increased BA concentration increased multiplication rates (HUSSEY, 1976; HUSSEY, 1980; CUSTERS & BERGERVOET, 1992; DE BRUYN *et al*., 1992). The number of shoots formed by bulblets increased as the concentration of BA in the growth medium increased (FENNELL, 2002).

Materials and Methods

Bulblets which formed from twin-scales were excised from the twin-scales and their top two thirds were removed. Bulblets were either placed in tissue culture at this point (whole) or they were dissected in half longitudinally (half).

The medium was supplemented with 0 mgl^{-1}, 0.1 mgl^{-1} BA, 1 mgl^{-1} BA and 10 mgl^{-1} BA. Half and whole bulblets were placed on the sterilized MS. The culture conditions were as stated in the Materials and Methods on page 55.

Results

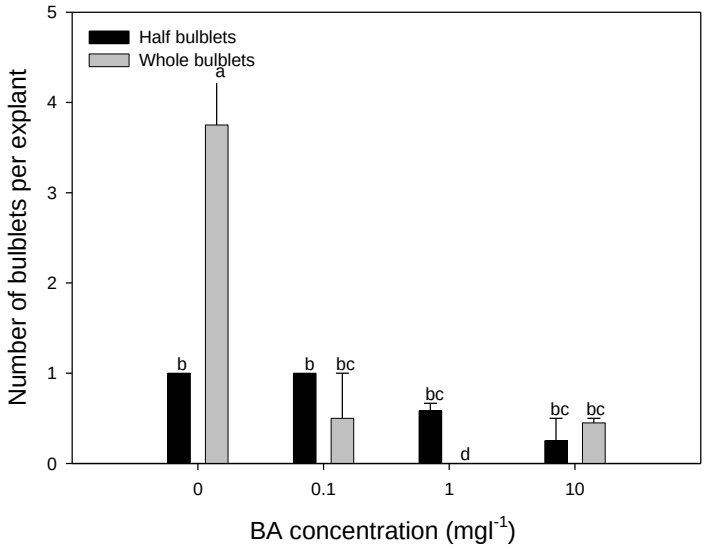


Figure 3.14: The effect of BA concentration on bulblet multiplication by whole and half-bulblets. Bars indicate standard error. Different letters indicate significant differences between treatments at the 5% level (ANOVA).

Figure 3.14 shows the number of bulblets which were formed by whole bulblets and half-bulblets placed on different concentrations of BA. The greatest multiplication was recorded for whole bulblets placed on hormone-free medium, where each bulblet formed a further 3.8 bulblets. Half-bulblets on hormone-free medium and medium supplemented with 0.1 mgl^{-1} BA formed 1 bulblet per half-bulblet. Whole bulblets on 0.1 mgl^{-1} BA formed 0.5 bulblets per bulblet, half-bulblets on 1 mgl^{-1} BA formed 0.6 bulblets per half-bulblet and whole bulblets and half-bulblets on 10 mgl^{-1} BA formed 0.4 and 0.3 bulblets respectively. These differences between treatments were not significantly different. No multiplication was observed for whole bulblets on 1 mgl^{-1} BA.

Discussion

The inclusion of BA in the growth medium decreased the multiplication rate of whole bulblets. Whole bulblets on hormone-free medium had a high multiplication rate which

was significantly decreased when BA was added to the medium at a concentration of 0.1 mgl^{-1}.

A similar trend was found for multiplication from half-bulblets. When 0.1 mgl^{-1} BA was present the multiplication rate was the same as in the control. The inclusion of BA in the medium at higher concentrations (1 mgl^{-1} and 10 mgl^{-1}) decreased multiplication.

Other bulbous species such as *Lilium* and *Narcissus* required BA concentrations of 2 mgl^{-1} and greater to induce multiplication (HUSSEY, 1976). Members of the Iridaceae are sensitive to BA concentration and require BA concentrations as low as 0.03 mgl^{-1} for multiplication. Multiplication from bulblets may be increased with the use of very low concentrations of BA in the growth medium.

The inclusion of BA in the growth medium does not enhance the *in vitro* multiplication of bulblets from *Brunsvigia undulata*.

3.1.7.2 The effect of photoperiod on multiplication

Introduction

Light conditions can influence bulblet differentiation (WITOMSKA & LUKASZEWSKA, 1997) and growth (FENNELL, 2002) *in vitro*. However the effects of light and dark conditions on bulblets are species specific. There have been reports of increased multiplication by continuous light (LESHEM *et al*., 1982; PIERIK *et al*., 1990; MAESATO *et al*., 1994), continuous dark (WITOMSKA & LUKASZEWSKA, 1997) and 16 h photoperiod (MORÁN *et al*., 2003). In other cases, photoperiod had no effect on bulblets (ALDERSON *et al*., 1986; ULRICH *et al*., 1999).

As bulblets are naturally accustomed to underground conditions, it is suggested that dark conditions increase bulblet growth and proliferation as this resembles their natural growth

conditions (ULRICH *et al*., 1999). While light increases the uptake and use of sugars (NIIMI *et al*., 2000) from the medium.

Materials and Methods

Bulblets which formed from twin-scales were excised from the twin-scales and their top two thirds were removed. Bulblets were either placed in tissue culture at this point (whole) or they were dissected in half longitudinally (half). Half and whole bulblets were placed on the sterilized MS. Cultures were placed in a 16 h photoperiod, under continuous light conditions and under continuous dark conditions. Fifteen explants were cultured per treatment. All other culture conditions were as stated in the Materials and Methods on page 55.

Results

The bulblets which formed under continuous dark conditions were white while those which grew in the 16 h photoperiod were green.

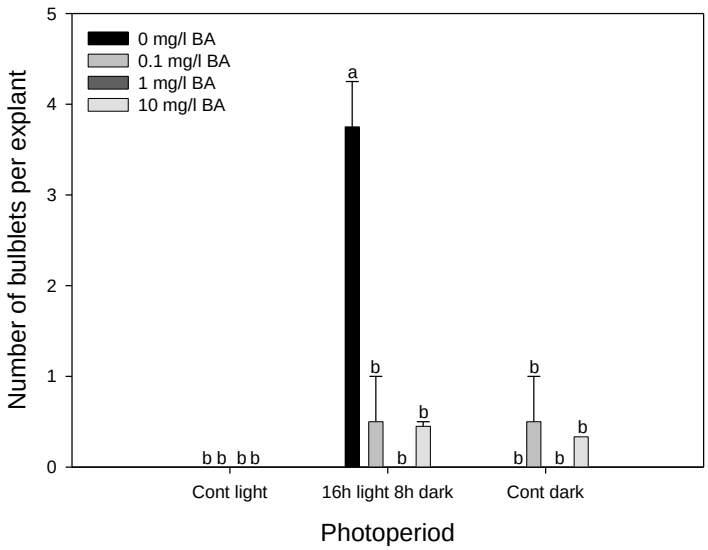


Figure 3.15: The effect of BA concentration and photoperiod on bulblet multiplication by whole bulblets. Bars indicate standard error. Different letters indicate significant differences between treatments at the 5% level (ANOVA).

Figure 3.15 shows the number of bulblets produced by whole bulblets on different concentrations of BA and kept in different photoperiods. No multiplication was observed by bulblets kept under continuous light. Similarly, bulblets on 1 mgl^{-1} BA in a 16 h photoperiod and those on hormone-free medium and on 1 mgl^{-1} BA in continuous dark, did not multiply. The highest multiplication was observed for bulblets on hormone-free medium, in a 16 h photoperiod where 3.8 bulblets were formed. Bulblets on 0.1 mgl^{-1} BA both in a 16 h photoperiod and in continuous dark formed 0.5 bulblets per bulblet. Bulblets on 10 mgl^{-1} BA in a 16 h photoperiod and in continuous dark formed 0.5 and 0.33 bulblets respectively. The differences between these four treatments were not significantly different.

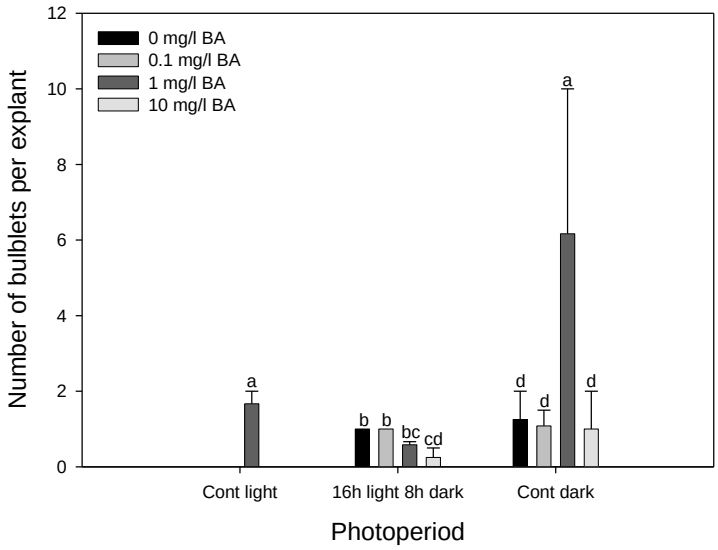


Figure 3.16: The effect of BA concentration and photoperiod on bulblet multiplication by half-bulblets. Bars indicate standard error. Different letters indicate significant differences between treatments at the 5% level (ANOVA).

Figure 3.16 shows the number of bulblets formed by half-bulblets on different concentrations of BA and in different photoperiods. The greatest multiplication was observed by half-bulblets on 1 mgl^{-1} BA in the continuous dark, where 6.2 bulblets were formed per explant. Half-bulblets on 1 mgl^{-1} BA and in continuous light formed 1.7 bulblets per explant. Bulblet formation in these treatments was significantly higher than

in the other treatments. These two treatments were not significantly different from each other.

Half-bulblets in a 16 h photoperiod on both hormone-free medium and on 0.1 mgl^{-1} BA formed one bulblet per explant. Half-bulblets in a 16 h photoperiod on 1 mgl^{-1} BA and on 10 mgl^{-1} BA formed 0.6 and 0.3 bulblets per explant, respectively. These differences were not significant from each other. Half-bulblets on hormone-free medium, 0.1 mgl^{-1} BA and 10 mgl^{-1} BA in continuous dark formed 1.3 bulblets, 1.1 bulblets and 1 bulblet per explant. These differences are not significant from each other. Continuous dark conditions are best for the multiplication of half-bulblets of *Brunsvigia undulata*.

Discussion

In general, continuous light inhibited multiplication of whole bulblets and resulted in poor multiplication by half-bulblets. These results are consistent with those obtained for the Liliaceae (LESHEM *et al*., 1982) and *Narcissus* (STEINITZ & YAHEL, 1982) where bulblet growth was limited by light. Half-bulblets had greater multiplication rates in continuous dark than in 16 h photoperiod. Bulblet formations is stimulated by darkness (VAN AARTRIJK & VAN DER LINDE, 1986). Bulblets are underground organs, and so they grow in dark conditions (ULRICH *et al*., 1999). Exposure to continuous light would hinder bulblet multiplication as bulblets are accustomed to growing in dark conditions. This may explain the poor multiplication of both whole and half-bulblets in continuous light conditions.

Half-bulblets growing in a 16 h photoperiod showed greater multiplication than those growing in continuous light but not as successful as those growing in continuous dark conditions. Darkness induced shoot formation in *Crinum moorei* cultures whereas light was required for bulblet formation (FENNELL, 2002). This may be related to the number of hours of darkness to which the explants were exposed. The longer the period of darkness the greater the multiplication rate of half-bulblets.

There was no significant difference between multiplication of whole bulblets in a 16 h photoperiod and in continuous dark, except for the whole bulblets on hormone-free medium in a 16 h photoperiod. The increased multiplication of this treatment may be due to the ability of the explant to use sugars from the medium. Light aids in the uptake and use of sugars (NIIMI *et al*., 2000). Exposure to a dark period increased the multiplication by whole bulblets in the 16 h photoperiod and continuous dark compared with the multiplication observed in the continuous light. Darkness resembles the natural growth conditions of bulblets.

Half-bulblets had higher multiplication rates than whole bulblets under all light conditions. Multiple shoots and bulblets form from the basal plate tissue of the explant. When bulblets are dissected in half their inner tissues are exposed to the external conditions and so the response of the internal tissue will be determined by the external conditions. The scales of half-bulblets are free to move apart from each other, allowing for multiple shoots and bulblets to form between the scales. The surface area for multiplication is greater in half-bulblets than in whole bulblets. The inner scales of the half-bulblets are not usually exposed to light, and so half-bulblets responded best to continuous dark conditions.

3.1.7.3 The effect of temperature on multiplication

Introduction

Culture conditions for bulbous plants, in particular temperature, play an important role in their growth and development *in vitro* (MOCHTAK, 1989). Temperature affects the uptake and use of sugars (NIIMI *et* al., 2000). An increase in the uptake of sugars will generate an increase in the growth and development of the explant in culture.

The most common temperature at which to keep tissue cultures is 25 °C. This is the optimum temperature for many bulbous species such as *Lilium* (CHANG *et al*., 2000), *Fritillaria* (PAEK & MURTHY, 2002), *Lycoris* (HUANG & LIU, 1989) and *Crinum*

(SLABBERT *et al*., 1993). YEOMAN (1986) suggest, however, that the optimum temperature for bulbous species is often lower than 25 °C. For this reason it is best to establish the optimum temperature for the tissue culture of a specific species. This will aid in achieving the highest regeneration from the cultures.

Materials and Methods

The medium was supplemented with 0, 0.1, 1 and 10 mgl^{-1} BA. Half and whole bulblets were placed on the sterilized MS. Cultures were kept at 15 °C, 20 °C, 25 °C and 30 °C. Fifteen explants were cultured in each treatment. All other culture conditions were as stated in the Materials and Methods on page 55.

Results

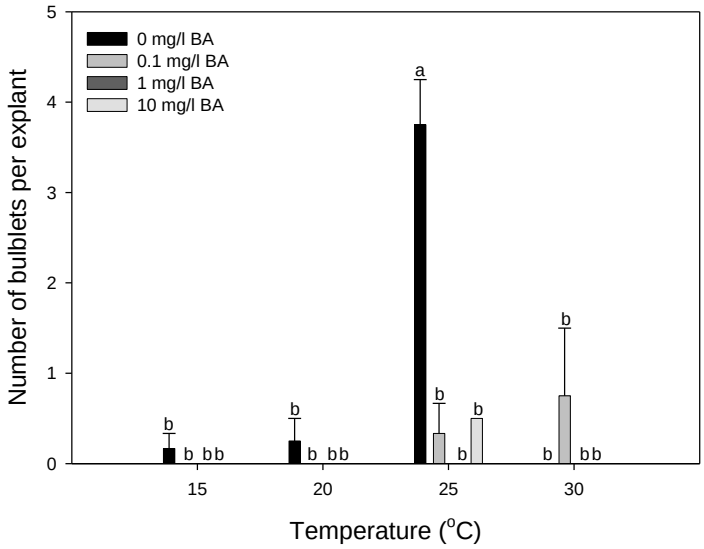


Figure 3.17: The effect of BA concentration and temperature on bulblet multiplication by whole bulblets. Bars indicate standard error. Different letters indicate significant differences between treatments at the 5% level (ANOVA).

Figure 3.17 shows the number of bulblets produced by whole bulblets on different concentrations of BA and kept at different temperatures. The greatest multiplication was achieved by bulblets on hormone-free medium at 25 °C.

Bulblets on hormone-free medium at 15 °C and 20 °C formed 0.2 and 0.3 bulblets per explant, respectively. Bulblets on other treatments at these temperatures did not multiply. Bulblets on 0.1 mgl^{-1} BA at 25 °C and 30 °C formed 0.3 and 0.8 bulblets, respectively. Bulblets on other treatments at these temperatures did not multiply. The differences between these treatments were not significant.

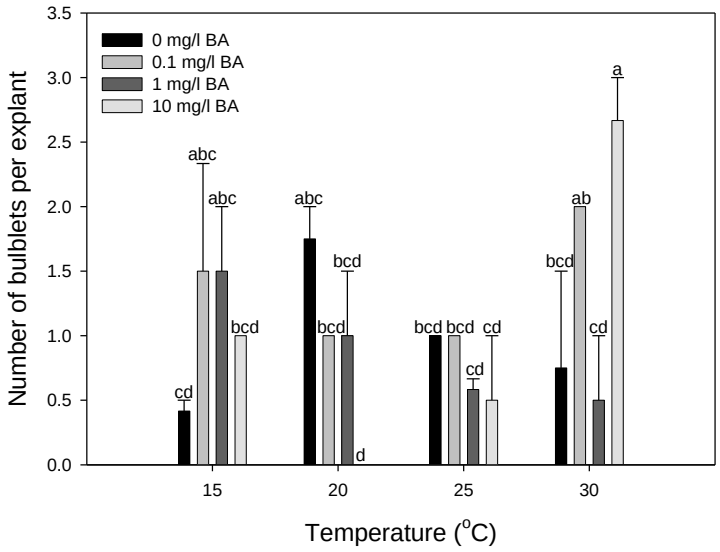


Figure 3.18: The effect of BA concentration and temperature on bulblet multiplication by half-bulblets. Bars indicate standard error. Different letters indicate significant differences between treatments at the 5% level (ANOVA).

Figure 3.18 shows the number of bulblets formed by half-bulblets on different concentrations of BA and kept at different temperatures. Bulblets were formed with all treatments except one, 10 mgl^{-1} BA at 20 °C. The greatest multiplication was seen with 10 mgl^{-1} at 30 °C, where 2.7 bulblets were formed. Explants on 0.1 mgl^{-1} BA at 30 °C formed 2 bulblets per explant.

Explants on hormone-free medium at 20 °C formed 1.8 bulblets per explant. Explants on 0.1 mgl^{-1} BA and 1 mgl^{-1} BA at 15 °C formed 1.5 bulblets per explant. The differences between these treatments are not significant.

Discussion

The greatest multiplication of whole bulbs was observed at 25 °C on hormone-free medium (Figure 3.17). Bulblet production for the multiplication of *Narcissus* was highest at 25 °C when compared to cultures maintained at 15 °C, 20 °C and 30 °C (SEABROOK & CUMMING, 1982). Bulblet development in *Nerine* was stimulated at 25 °C (PIERIK & IPPEL, 1977). This was also the optimum temperature for cultures of *Crinum moorei* (FENNELL, 2002) and *Narcissus* (STEINITZ & YAHEL, 1982). It was suggested that the optimum temperature for the tissue culture of a species will be related to the temperature of its natural habitat (FENNELL, 2002). Bulbous species from warmer areas would require warmer culture temperatures for optimal growth. High temperatures stimulate the use of accumulated carbohydrates, due to higher respiration at high temperatures (FENNELL, 2002). This will supply the explant with more energy and so greater multiplication can take place.

Whole bulblets cultured at temperatures both lower and higher than 25 °C showed poor multiplication. There were no significant differences between the multiplication rates at these temperatures.

The greatest multiplication from half-bulblets was observed at 30 °C on 10 mgl^{-1} BA (Figure 3.18). Generally multiplication of half-bulblets was best at 30 °C. On medium supplemented with 0.1 mgl^{-1} BA multiplication was greatest at 30 °C. Temperatures as high as 30 °C stimulate bulbing (VAN AARTRIJK & VAN DER LINDE, 1986). Low temperatures increase endogenous levels of GA which inhibits bulblet production (HANKS *et al*., 1986).

Overall, half-bulblets generally performed better than whole bulblets at all temperatures. Half-bulblets had a greater surface area for the production of multiple shoots and bulblets.

3.1.7.4 The effect of activated charcoal on multiplication

Introduction

Activated charcoal is often added to tissue culture medium as it has beneficial effects for the growth and development of explants. Activated charcoal has been reported to enhance bulblet growth (FENNELL, 2002) and regeneration (STEINITZ & YAHEL, 1982) *in vitro*.

As mentioned previously, activated charcoal adsorbs inhibitory compounds that may be in the medium, as a result of autoclaving (PAN & VAN STADEN, 1998) or polyphenols released by the explant (WEATHERHEAD *et al*., 1978; PAN & VAN STADEN, 1998). Activated charcoal also binds to plant growth hormones in the medium, rendering them inactive (WANG and HUANG, 1976).

Activated charcoal, when included in growth medium, causes darkening of the medium. This means that the medium simulates soil conditions (WANG & HUANG, 1976). As bulblets are soil-born plant organs this darkened medium will favour their growth and development (FENNELL, 2002).

Materials and Methods

To investigate the effect of activated charcoal on bulblet multiplication from whole or half-bulblets, the medium was supplemented with 0 gl^{-1} and 5 gl^{-1} activated charcoal. Half and whole bulblets were placed on the sterilized MS. The culture conditions were as stated in the Materials and Methods on page 55.

Results

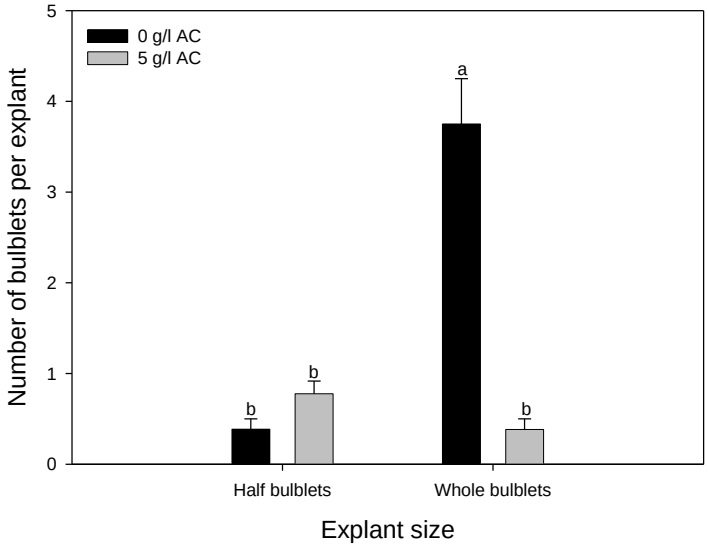


Figure 3.19: The effect of activated charcoal on bulblet multiplication by whole and half-bulblets. Bars indicate standard error. Different letters indicate significant differences between treatments at the 5% level (ANOVA).

Figure 3.19 shows the number of bulblets formed by half and whole bulblets placed on medium with and without activated charcoal. The greatest multiplication was observed by whole bulblets on medium without activated charcoal.

Half-bulblets on medium with activated charcoal formed 0.4 bulblets, while those on medium without activated charcoal formed 0.8 bulblets. Whole bulblets on medium without activated charcoal formed 0.5 bulblets. The differences between these treatments are not statistically significant.

Discussion

Although activated charcoal increased multiplication from half-bulblets, the differences between bulblet multiplication with and without activated charcoal are not statistically

significant. Multiplication by half-bulblets was not enhanced by the addition of activated charcoal to the growth medium.

The presence of activated charcoal significantly inhibited multiplication of whole bulblets when compared to multiplication on medium without activated charcoal. Activated charcoal did not enhance multiplication by whole or half-bulblets of *Brunsvigia undulata*.

3.1.7.5 The effect of sucrose concentration on multiplication

Introduction

Sucrose is essential for shoot production (FENNELL, 2002) and regeneration (DE BRUYN *et al*., 1992). Bulb formation is stimulated by sucrose in bulbous plants (CHOW *et al*., 1992; DE BRUYN & FERREIRA, 1992). *In vitro* organogenesis and the rate of regeneration are regulated by sucrose (TAEB & ALDERSON, 1990).

High sucrose levels are required for bulblet growth *in vitro* and low sucrose concentrations have been shown to hinder the onset of bulbing (VAN AARTRIJK & VAN DER LINDE, 1986). For all other experiments in this study the growth medium used had a 3% sucrose concentration. An increase in sucrose concentration may increase multiplication.

Materials and Methods

To investigate the effect of sucrose concentration on bulblet multiplication, medium was supplemented with 0%, 2%, 4%, 6% and 8% sucrose. Half and whole bulblets were placed on the sterilized MS. Fifteen explants were cultured per treatment. The culture conditions were as stated in the Materials and Methods on page 55.

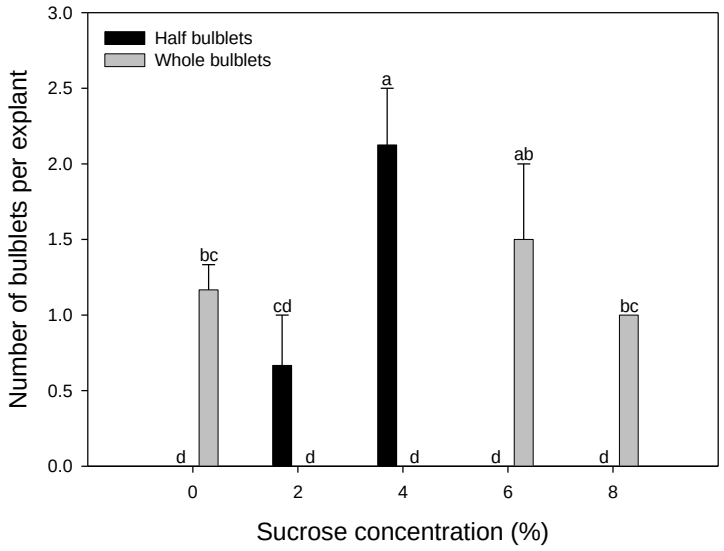


Figure 3.20: The effect of sucrose concentration on bulblet multiplication by whole and half-bulblets. Bars indicate standard error. Different letters indicate significant differences between treatments at the 5% level (ANOVA).

Figure 3.20 shows the number of bulblets formed when whole and half-bulblets were placed on medium supplemented with different sucrose concentrations. The greatest multiplication was achieved by half-bulblets on medium with 4% sucrose, where 2.1 bulblets were formed per explant. Whole bulblets on medium with 6% sucrose formed 1.5 bulblets per explant.

Whole bulblets on medium without sucrose formed 1.1 bulblets per explant, and those on medium with 8% sucrose formed 1 bulblet per explant. The differences between these two treatments are not statistically significant.

Half-bulblets on medium with 2% sucrose formed 0.6 bulblets per explant. Multiplication was not seen by half-bulblets on medium without sucrose, medium with 6% sucrose and medium with 8% sucrose. Multiplication was not achieved by whole bulblets on medium with 2% sucrose and medium with 4% sucrose.

Discussion

Multiplication from half-bulblets increased in the presence of 2% and 4% sucrose. At higher levels, however, multiplication was inhibited by higher sucrose concentrations. The optimum sucrose concentration for *Crinum moorei* was 4% (FENNELL, 2002). As bulblets are storage organs, they have high starch reserves. It follows that low concentrations of sucrose are needed to initiate shoots (VAN AARTRIJK & BLOM-BARNHOORN, 1980). When the sucrose concentration in the medium is increased so too is the osmotic potential of the medium. This can limit the growth of bulblets (DANTU & BHOJWANI, 1995), by causing the bulblets to take up less water from the medium. In addition to the high sucrose concentrations of between 6% and 9% has been shown to cause the early onset of dormancy (SQUIRES *et al*., 1991).

Multiplication of whole bulblets was increased by high sucrose concentrations, but inhibited by low sucrose concentrations. SELLÉS *et al*. (1997) found that 9% sucrose was the optimum concentration for multiplication of *Narcissus*. For bulblet multiplication 9% sucrose is added to the growth medium (SANTOS *et al*., 1998; NIIMI *et al*., 2000). Higher sucrose concentrations resulted in greater multiplication of bulblets (LIAN *et al*., 2003). Bulblet formation from shoot clumps was increased by increasing the sucrose concentration in the medium (CHOW *et* al., 1992). Sucrose is the main sugar for the storage of starch which occurs in regions which form shoots (VAN AARTRIJK & BLOM-BARNHOORN 1980). An accumulation of starch in these regions may increase the number of shoots formed and so increase the multiplication rate.

The multiplication from half-bulblets of *Brunsvigia undulata* is enhanced by low sucrose concentrations, while multiplication from whole bulblets is enhanced by high sucrose concentrations.

3.1.7.6 The effect of liquid culture on multiplication

Introduction

The use of liquid culture has been reported to increase the rate of proliferation of propagules (ZIV, 1989; WATAD *et al*., 1995; ZIV & LILIEN-KIPNIS, 1997; NIIMI *et al*., 2000; FENNELL, 2002). These effects can be attributed to the better availability of nutrients to the explants in liquid culture. There is a greater water potential and diffusion rate in a liquid culture system (GEORGE, 1993). Half-bulblets of *Crinum moorei* formed meristematic clusters when cultured in liquid culture supplemented with different concentrations of BA (FENNELL, 2002).

Materials and Methods

A liquid medium was prepared by excluding agar and adding 0 mgl^{-1}, 0.1 mgl^{-1} BA, 1 mgl^{-1} BA and 10 mgl^{-1} BA. As a control, medium with agar was also prepared. Half- and whole bulblets were placed in culture vessels containing liquid medium which were sealed with cotton wool plugs and aluminium foil. These cultures were maintained on a rotary shaker at 124 rpm. All other culture conditions were as stated in the Materials and Methods on page 55 and half- and whole bulblets were also cultured on solid medium as previously described.

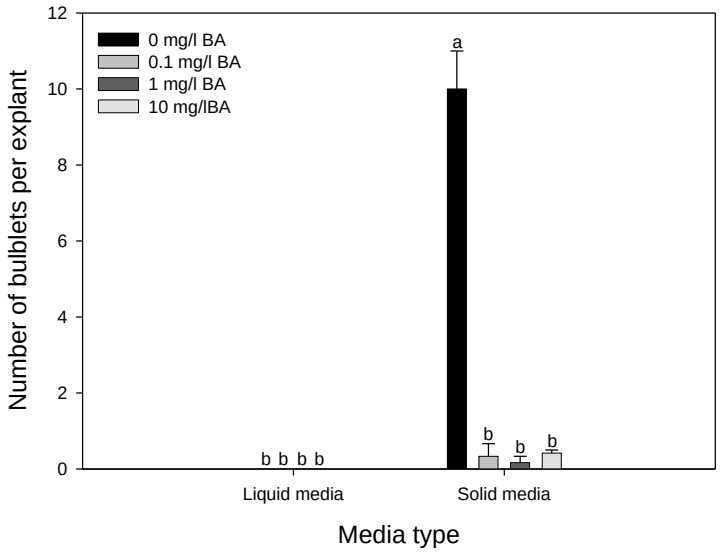


Figure 3.21: The effect of BA concentration and medium type on bulblet multiplication by whole bulblets. Bars indicate standard error. Different letters indicate significant differences between treatments at the 5% level (ANOVA).

Figure 3.21 shows the number of bulblets produced by whole bulblets placed on solid and in liquid medium. The bulblets placed in liquid medium grew in size but did not form secondary bulblets or shoots. On solid medium, bulblets placed on the hormone-free medium had the highest bulblet regeneration, producing 10.6 bulblets per explant.

Bulblets placed on the solid medium supplemented with different concentrations of BA formed bulblets but at a very low rate. The differences between the multiplication rates of these treatments were not significant.

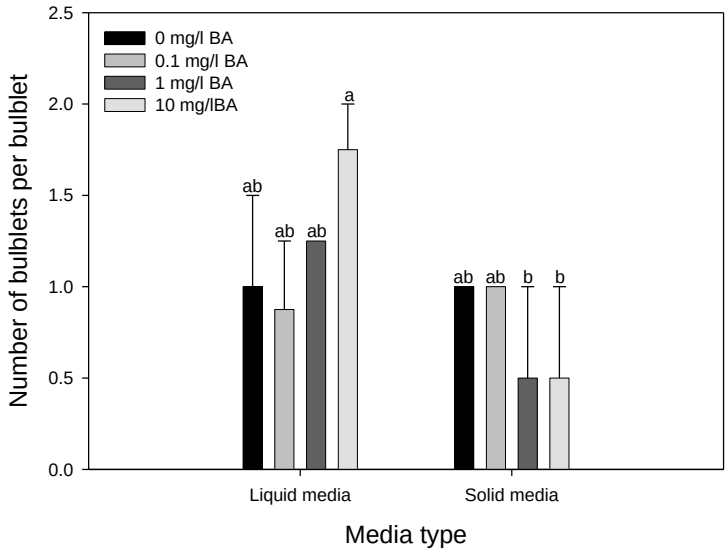


Figure 3.22: The effect of BA concentration and medium type on bulblet multiplication by half-bulblets. Bars indicate standard error. Different letters indicate significant differences between treatments at the 5% level (ANOVA).

Figure 3.22 shows the number of bulblets which were produced by half-bulblets placed on solid and in liquid medium containing different concentrations of BA. The greatest number of bulblets produced was observed by half-bulblets in liquid culture, supplemented with 10 mgl^{-1} BA, where 1.8 bulblets formed per explant.

Explants in liquid culture supplemented with lower and intermediate concentrations of BA and explants on solid medium supplemented with no or low concentrations of BA formed similar numbers of bulblets. Explants on solid medium supplemented with higher concentrations of BA produced few bulblets per explant.

Discussion

When whole bulblets were placed in liquid culture, multiplication was inhibited (Figure 3.21). Greater bulblet production was observed on solid medium (LIAN *et al*., 2003). Bulblets may have accumulated excess water from the medium. This would lead to oxygen depletion in the cells (CHEN & ZIV, 2001), inhibiting multiplication. As bulblets

in liquid culture are submerged there is a risk that the culture will not be properly aerated. Explants on solid medium have support from the medium which allows for high levels of aeration (PRASAD & DUTTA GUPTA, 2006). Explants turned brown in liquid culture and were damaged by leaching into the medium (LIAN *et al.*, 2003).

In liquid medium without hormones and in medium supplemented with 0.1 mgl^{-1} BA multiplication rates from half-bulblets were not significantly affected by liquid culture (Figure 3.22). ANGULO *et al*. (2003) reported that there was a biomass gain for bulblets in liquid culture, but no increase in multiplication.

In medium supplemented with 1 mgl^{-1} BA and 10 mgl^{-1} BA liquid culture increased multiplication from half-bulblets. Liquid cultures allowed for more rapid multiplication (ZIV, 1989; WATAD *et al*., 1995; FENNELL, 2002). Bud clusters increased 4-fold when placed in liquid culture (ZIV & LILIEN-KIPNIS, 1997). In liquid culture the explant is surrounded by the nutrient medium. This allows for a greater uptake of nutrients (GEORGE, 2008). In addition, the nutrients are at the same concentration at all points in the medium allowing all parts of the explant to have access to the same concentrations of nutrients. There is no gradient in nutrient concentration in liquid medium (BERGOÑÓN *et al*., 1992) as may develop in solid medium. This allows for greater proliferation in liquid culture.

CONCLUSIONS

Twin-scales were successful when used as explants for bulblet production of *Brunsvigia undulata*. Bulblet production was however quite low from this explant type. Using bulbous explants, requires the collection and destruction of the entire plant.

For the best results when using twin-scales as explants for bulblet production, twin-scales excised from the middle of the parent bulb should be used. These should be place adaxial side down on hormone-free medium and kept in a 16 h photoperiod.

Approximately 100 twin-scales may be excised from a bulb of *Brunsvigia undulata* with a diameter of ±10 cm. If each twin-scale only formed one bulblet each, 100 bulblets could be produced from one parent bulb. If each bulblet is split in half, 200 half-bulblets can be put into culture for multiplication. Under optimum conditions (in continuous dark and on 1 mgl^{-1} BA) each of these half-bulblets could form a further 6.2 bulblets each, resulting in 1240 bulblets from one parent bulb.

In general, half-bulblets had a higher multiplication rate than whole bulblets in the multiplication experiments. Half-bulblets have been reported to be more productive than whole bulblets (STEINITZ & YAHEL, 1982; SLABBERT *et al*., 1993; FENNELL, 2002). Dormant *Freesia* bulblets will begin to grow again if their tops are removed and they are cut in two (HUSSEY, 1976).

Dormancy was broken when the shoots and the tops of the whole bulblets were removed. However, apical dominance was not broken as the basal plate was not cut. Apical dominance was exerted by the basal plate while whole bulblets were in culture and so there was poor axillary shooting. This will explain why half-bulblets were more successful in the multiplication experiments than whole bulblets.

For multiplication, bulblets must form from the basal plate tissue of the whole of half-bulblet in culture. When bulblets are dissected their inner scales are free to move apart from each other. This allows bulblets to form from the basal plate tissue between the scales. Thus half-bulblets have a greater surface area than whole bulblets for the formation of further bulblets.

From the results of the multiplication experiments it is clear that the multiplication of whole bulblets was hindered by the inclusion of BA in the medium. Multiplication by whole bulblets was less on medium supplemented with BA than on the control medium. These results suggest that BA inhibits multiplication by whole bulblets. MII *et al*. (1974) found that cytokinins caused explant death and, suggested that cytokinins may be toxic to bulb explants. Bulb tissue may already contain high endogenous levels of cytokinin and

the added cytokinin in the medium means that the explant is exposed to toxic levels of cytokinin (MII *et al*., 1974). Half-bulblets may not be affected by the cytokinin in the medium in quite the same way. Perhaps the more open structure of the explant allows for endogenous cytokinins to be leached into the medium.

In conclusion it is favourable to split bulblets in half for multiplication experiments. This will yield more explants and a greater multiplication of propagules.

3.2 TISSUE CULTURE FROM LEAF AND FLOWER EXPLANTS

MATERIALS AND METHODS

Figure 3.23 shows an overview of the methods used to establish tissue cultures using leaf and flower explants. Leaves were collected and 1x1 cm leaf explants were placed in culture.

Anthers, filament sections, ovaries, ovules and sections of the pedicel were excised from unopened flowers buds collected.

Unless otherwise stated the growth medium used was MS (MURASHIGE & SKOOG, 1962) with 0.1 gl^{-1} *myo*-inositol, 8 gl^{-1} agar (Agar Bacteriological-Agar No. 1, Oxoid Ltd., England) and 3% sucrose. The culture medium was sterilized by autoclaving for 20 min at 121 °C and 103.4 kPa.

Explants were placed on the medium and culture tubes were sealed with metal caps and Parafilm®. Cultures were kept at 25 ±1 °C under Osram® 75 W cool white fluorescent tubes in a 16 h photoperiod with a light intensity of 34.2 µmol m^{-2} s^{-1}.

Figure 3.23

Leaf explants and floral explants did not successfully form bulblets in culture. Leaf explants formed callus along their cut edges and then turned brown. There was limited success from leaf explants in culture (SLABBERT *et al*., 1993; CHAUDHURI & SEN, 2002). FENNELL and VAN STADEN (2004) reported that "only bulbs and inflorescence stems are productive in the Iridaceae and Amaryllidaceae". Although leaf explants produced callus this was in response to wounding. *Crinum moorei* leaf explants formed crystalline callus as a wounding response (FENNELL, 2002). The callus produced was too delicate to sub-culture.

Floral explants were not successful in culture as explants may have been damaged by decontamination. The peduncle of *Crinum moorei* was a successful source of explants for tissue culture (FENNELL, 2002). Peduncle tissue may be a successful source of explants for tissue culture of *Brunsvigia undulata*.

3.3 ACCLIMATIZATION

Introduction

Transferring propagules to the natural environment is the final stage in micropropagation. Some view this as the most important stage as if plantlets cannot survive *ex vitro* there is little use for the plantlets formed *in vitro*. Often this is the hardest stage to perfect.

The advantage of obtaining bulblets as the final product of a tissue culture protocol is that they are relatively easy to acclimatize. Bulblets do not need a rooting stage (FENNELL *et al*., 2001). Theoretically bulblets may be sown like seed.

Materials and Methods

Once bulblets had a diameter of 3 mm or more and had at least one root they were removed from culture. All medium was washed from the bulblets and their roots with distilled water. Bulblets were subsequently rinsed in 1% Benlate for 5 min.

Rinsed bulblets were planted in a 1:1 mixture of perlite and vermiculite. This planting mixture was watered with 1% Benlate. Bulblets in trays were placed in a mist house at the botanical gardens at the University of KwaZulu-Natal, Pietermaritzburg for 14 days. The mist house was kept at a temperature of 22 ± 2 °C and at a relative humidity of 85%. Once bulblets had acclimatized they were moved to a green house and placed alongside a wet wall for a further 14 days, at a temperature of 24 ± 1 °C.

Results

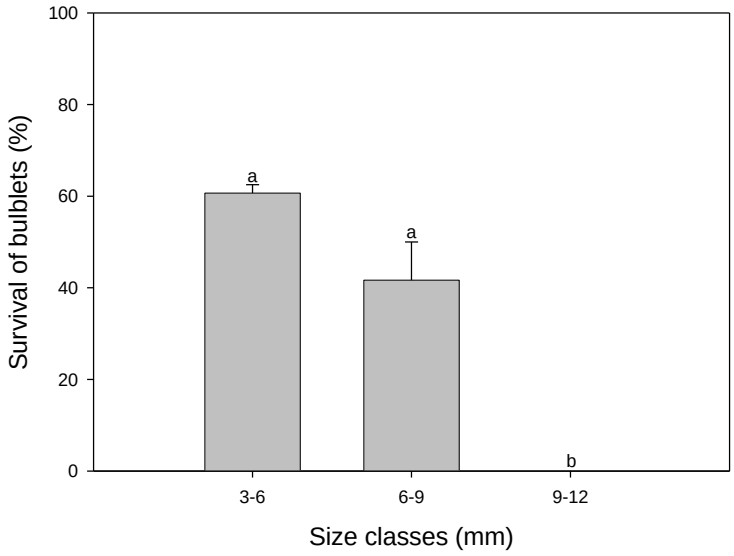


Figure 3.24: Percentage survival of bulblets in different size classes after 42 days *ex vitro*. Bars indicate standard error. Different letters indicate significant differences between treatments at the 5% level (ANOVA).

Figure 3.24 shows the percentage of bulblets in different size classes which survived after 42 days *ex vitro*. Small bulblets (3-6 mm) had the greatest percentage survival, while medium-sized bulblets (6-9 mm) had a slightly lower survival rate. The differences between these two size classes are not statistically significant. None of the larger bulblets survived *ex vitro*.

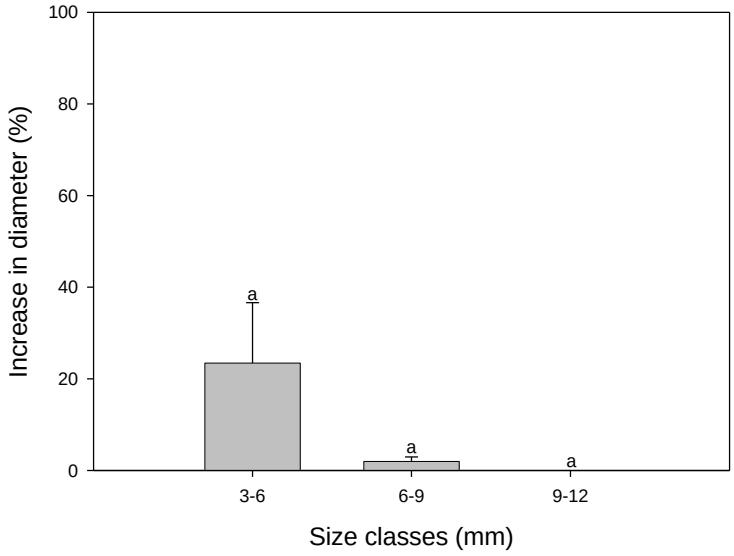


Figure 3.25: Increase in diameter of bulblets in different size classes after 42 days *ex vitro*. Bars indicate standard error. Different letters indicate significant differences between treatments at the 5% level (ANOVA).

Figure 3.25 shows the increase in diameter by bulblets in different size classes after 42 days *ex vitro*. The greatest increase in diameter was seen by bulblets in the smallest size class. Poor increase in diameter was observed by medium sized bulblets. Large bulblets did not survive. The differences between the three size classes is not statistically significant

Discussion

Smaller bulblets survived acclimatization (Figure 3. 24) and showed the greatest increase in diameter (Figure 3.25). The survival of bulblets *ex vitro* depends on their size (DRAGASSAKI *et al*., 2003). The mist house provides a very moist environment (CHANG *et al*., 2000). Larger bulblets will take up more water than smaller bulblets while in the mist house. This may increase the potential for larger bulblets to rot and die.

Chapter 4

CONCLUSIONS

Four possible sources of explants were investigated for use in *in vitro* bulblet production of *Brunsvigia undulata*. Explants from seeds, twin-scales, leaves and flowers were put into culture to establish whether or not they would form bulblets.

The use of explants from seedlings derived from seeds germinated *in vitro* was successful in producing bulblets of *Brunsvigia undulata*. Twin-scales were also a successful explant in producing bulblets *in vitro*.

Seedling sections readily formed bulblets, shoots and callus when a meristematic region was present in the explant. When sub-cultured, callus formed shoot clusters comprised of many shoots. Callus was sub-cultured for a second time and still formed shoot clusters, although they were not as prolific as previously.

Twin-scales formed bulblets even in the absence of plant growth regulators. The greatest number of bulblets formed per twin-scale was quite low, only 2.5 bulblets. Multiplication of these bulblets was slow and although the multiplication rate was as high as 10.6 bulblets per explant in one treatment (solid medium without hormones), multiplication in all other treatments was considerably lower. Activated charcoal did not enhance bulblet production by twin-scales and bulblet formation was more successful when twin-scales are cultured in a 16 h photoperiod.

Using both seedling sections and twin-scales requires very little hormones. This means that they are both relatively cheap tissue culture protocols. Bulblet initiation from twin-scales is a little more time consuming as the scales are often difficult to decontaminate, separate and excise.

The multiplication by bulblets derived from twin-scales was much lower than those derived form seedling sections. Bulblets from seedling sections multiplied on all treatments while bulblets from twin-scales did not. FENNELL (2002) found that the growth of sub-cultured bulblets derived from twin-scales was slow. Multiplication of bulblets from seedling sections was quicker than multiplication of bulblets from twin-scales. Firstly, bulblets on seedling sections and those on twin-scales would have developed differently as they formed from different tissue types. "Cultures established from different parts of the same plant may also differ in their pattern of development" (YEOMAN, 1986). Secondly, the tissues in the seedling sections were younger than the twin-scale tissues. Younger tissues will have greater regeneration potential. Finally seedlings grew from decontaminated seeds. The seedling tissue did not come into contact with the decontaminating agent and so the seedling was not damaged in any way. Twin-scale tissue came into contact with the decontaminating agent during decontamination of the parent bulb. During this process the twin-scale tissue may have been damaged. This would have decreased the number of bulblets which were formed on the twin-scales.

There are no meristematic cells in the axils of very young bulblets (GROOTAARTS *et al*., 1981). Small bulblets also lack sufficient scales and basal plate tissue (PIERIK & IPPEL, 1977). It is thought that perhaps bulblets were too young when they were removed from twin-scales and sub-cultured for multiplication. If bulblets had been left to grow a little larger and then been used for multiplication, there may have been greater success. CUSTERS and BERGERVOET (1992) used larger bulblets with more scales for the multiplication of *Nerine*. For secondary multiplication bulblets with a diameter of 5 mm or greater, dissected in half should be used (SLABBERT *et al.*, 1993).

Larger bulblets would also have the advantage of supplying more than two explants per bulblet, for example, DE BRUYN *et al*. (1992) dissected large bulblets into four explants for the multiplication of *Amaryllis belladonna*.

The collection of seeds does not damage the plant and is not as labour intensive as collecting bulbs. When bulbs are collected the whole plant is removed from the wild,

while when seeds are collected the plant is left behind to flower and set seed again in the next year. Removing bulbs from the ground is difficult as they are anchored by a deep root system. It is also easy to damage the bulbs when they are being dug up.

Leaves and flowers were poor sources of explants. These explants did not respond in culture. Further work will have to be carried out to refine culture conditions in which these explant types will respond.

In conclusion it is suggested that seedling-derived explants are used for the production and multiplication of bulblets of *Brunsvigia undulata*.

References

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

AFOLAYAN, A.J. & ADEBOLA, P.O. 2004. *In vitro* propagation: a biotechnological tool capable of solving the problem of medicinal plants decimation in South Africa. *African Journal of Biotechnology* **3**: 683-687.

ALDERSON, P.G. & RICE, R.D. 1986. Propagation of bulbs from floral stem tissues. In: Withers, L.A. & Alderson, P.G. (Eds.). 1986. *Plant Tissue Culture and its Agricultural Applications*. Butterworth, London. ISBN 0-407-00921-3.

ALDERSON, P.G., TAEB, A.G. & RICE, R.D. 1986. Micropropagation of Tulip: bulbing of shoots in culture. *Acta Horticulturae* **177**: 291- 298.

ANGULO, M.E., COLQUE, R., VILADOMAT, F., BASTIDA, J. & CODINA, C. 2003. *In vitro* production of bulblets of *Cyrtanthus loddigesianus* and *Cyrtanthus speciosus*. *Journal of Horticultural Science and Biotechnology* **78**: 441-446.

ASCOUGH, G.D., ERWIN, J.E. & VAN STADEN, J. 2008. *In vitro* storage organ formation of ornamental geophytes. *Horticultural Reviews* **34**: 417-444.

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

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

BERJAK, P., FARRANT, J.M., MYCOCK, D.J. & PAMMENTER, N.W. 1990. Recalcitrant (homoiohydrous) seeds: the enigma of their desiccation-sensitivity. *Seed Science Technology* **18**: 297-310.

BERLJAK, J. & BENEDICIC, D. 1997. *In vitro* multiplication and propagation of a Slovenian selection of shallot (*Allium ascalonicum* L. cv. Pohorka). *Acta Horticulturae* **447**:119-120.

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.

BOREK, S. & RATAJCZAK, W. 2002. Sugars as a metabolic regulator of storage protein mobilization in germinating seeds of yellow lupine (*Lupinus luteus* L.). *Acta Physiologia Plantarum* **24**: 425-434.

BRUNT, A.A. 1985. The production and distribution of virus-tested ornamental bulb crops in England: Principles, practice and prognosis. *Acta Horticulturae* **164**: 153-162.

CAMPBELL, W.E., NAIR, J.J., GAMMON, D.W., CODINA, C., BASTIDA, J., VILADOMAT, F., SMITH, P.J. & ALBRECHT, C.F. 2000. Bioactive alkaloids from *Brunsvigia radulosa*. *Phytochemistry* **53**: 587-591.

CHANG, C., CHEN, C.-T., TSAI, Y.-C. & CHANG, W.-C. 2000. A tissue culture protocol for propagation of a rare plant, *Lilium speciosum* Thunb. var. *gloriosoides* Baker. *Botanical Bulletin of Academia Sinica* **41**: 139-142.

CHAUDHURI, D. & SEN, S. 2002. *In vitro* response of *Scilla siberica*. *Scientia Horticulturae* **95**: 51-62.

CHEN, J. & ZIV, M. 2001. Ancymidol effects on oxidative stress and the regeneration potential of *Narcissus* leaves in liquid culture. *Acta Horticulturae* **560**: 299-302.

CHEN, J. & ZIV, M. 2006. The effects of storage condition on starch metabolism and regeneration potentials of twin-scales and inflorescence stem explants of *Narcisus tazetta*. *In vitro Cellular & Developmental Biology* **41**: 816-821.

CHOW, Y.N., SELBY, C., FRASER, T.W. & 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.

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

COLQUE, R., VILADOMAT, F., BASTIDA, J. & CODINA, C. 2002. Micropropagation of the rare *Eucrosia stricklandii* (*Amaryllidaceae*) by twin-scaling and shake liquid culture. *Journal of Horticultural Science & Biotechnology* **77**: 739-743.

COPELAND, L.O. & MCDONALD, M.B. 2001. *Principles of Seed Science and Technology*. *Fourth edition*. Springer, Berlin. ISBN 0-792-7322-7.

CROUCH, N.R., CHETTY, J., MULHOLLAND, D.A. & NDLOVU, E. 2002. Bulb alkaloids of the reputedly psychoactive *Brunsvigia radulosa* (Amaryllidaceae). *South African Journal of Botany* **68**: 86-89.

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.

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

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

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

DE HERTOGH, A.A. & 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.

DE SMET, P.A.G.M. 1996. Some ethnopharmacological notes on African hallucinogens. *Journal of Ethnopharmacology* **50**: 141-146.

DODDS, J.H. 1991. *In Vitro Methods for Conservation of Plant Genetic Resources*. Chapman & Hall, London. ISBN 0-412-33870-X.

DRAGASSAKI, M., ECONOMOU, A.S. & VLAHOS, J.C. 2003. Bulblet formation *in vitro* and plantlet survival *extra vitrum* in *Pancratium maritimum* L. *Acta Horticulturae* **616**: 347-352.

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

DUNSTAN, D.I. & SHORT, K.C. 1977. Improved growth of tissue cultures of the Onion, *Allium cepa*. *Physiologia Plantarum* **41**: 70-72.

DU PLESSIS, N. & DUNCAN, G. 1989. *Bulbous Plants of Southern Africa - A Guide to their Cultivation and Propagation*. Tafelberg Publishers LTD, South Africa. ISBN 06240-2659-0.

DYER, R.A. 1976. *The Genera of Southern African Flowering Plants, Volume 2: Gymnosperms and Monocotyledons*. Botanical Research Institute, Pretoria. ISBN 0621-02868-0.

ELGORASHI, E.E., STAFFORD, G.I., JÄGER, A.K. & VAN STADEN, J. 2006. Inhibition of [^{3}H]Citalopram binding to the rat brain serotonin transporter by Amaryllidaceae alkaloids. *Planta Medica* **72**: 470-473.

ELLIS, R.H. & ROBERTS, E.H. 1981. The quantification of ageing and survival in orthodox seeds. *Seed Science and Technology* **9**: 373-409.

EVIDENTE, A., CICALA. M.R., RANDAZZO, G., RICCIO, R., CALABRESE, G., LISO, R. & ARRIGONI, O. 1983. Lycorine-structure-activity relationships. *Phytochemistry* **22**: 2193-2196.

FENNELL, C.W. 2002. Micropropagation and Secondary Metabolite Production in *Crinum macowanii*. Ph.D. Thesis. University of Natal, Pietermaritzburg.

FENNELL, C.W. & VAN STADEN, J. 2004. Biotechnology of Southern African bulbs. *South African Journal of Botany* **70**: 37-46.

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

FINNIE, J.F. 1989. Tissue Culture of Selected Indigenous Monocotyledons. Ph.D. Thesis. University of Natal, Pietermaritzburg.

FINNIE, J.F. & VAN STADEN, J. 1989. *In vitro* propagation of *Sandersonia* and *Gloriosa*. *Plant cell, Tissue and Organ Culture* **19**: 151-158.

FURMANOWA, M. & OLEDZKA, H. 1981. Plant regeneration from excised bulb scale segments of *Zephyranthes robusta* Baker. *Acta Society Botany Polonaise* **50**: 399-404.

GÉMESNÉ, J.A., SIMON-SARKADI, L., VELICH, I. & VARRO, P. 1997. Studies of Non-ionic osmotic stress on bean (*Phaseolus vulgaris* L.) callus and seedlings cultures. *Acta Horticulturae* **447**: 455-456.

GEORGE, E.F. 1993. *Plant Propagation by Tissue Culture*. *Part 1*. *The technology*. Exegetics Limited, Edington. ISBN 0-9509325-4-X.

GEORGE, E.F. 2008. *Plant Propagation by Tissue Culture*. *3rd Edition, Vol.1*. *The background*. Springer, Dordrecht. ISBN 9781402050053.

GROOTAARTS, H., SCHEL, J.H.N., & 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.

HAN, B.H., YAE, B.W., YU, H.J. & PEAK, K.Y. 2005. Improvement of *in vitro* micropropagation of *Lilium* oriental hybrid 'Casablanca' by the formation of shoots with abnormally swollen basal plates. *Scientia Horticulturae* **103**: 351-359.

HAN, B.H., YU, H.J., YAE, B.W. & PEAK, K.Y. 2004. *In vitro* micropropagation of *Lilium longiflorum* 'Georgia' by shoot formation as influenced by addition of liquid medium. *Scientia Horticulturae* **103**: 39-49.

HANKS, G.R. 1986. *Narcissus* bulb morphology and twin-scale propagation. *Acta Horticulturae* **177**: 309-313.

HANKS, G.R. 1987. Effects of growth retardants on bulbil production by *Narcissus* twin-scales. *Annals of Applied Biology* **110**: 203-207.

HANKS, G.R. & REES, A.R. 1977. Growth regulator treatments to improve the yield of twin-scaled narcissus. *Scientia Horticulturae* **6**: 237-240.

HANKS, G.R., SHAIK, G., & 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.

HARDEGREE, S.P. & EMMERICH, W.E. 1990. Partitioning water potential and specific salt effects on seed germination of four grasses. *Annals of Botany* **66**: 587-595.

HOL, G.M.G.M. & 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.

HOUGHTON, P.J., AGBEDAHUNSI, J.M. & ADEGBULUGBE, A. 2004. Choline esterase inhibitory properties of alkaloids from two Nigerian *Crinum* species. *Phytochemistry* **65**: 2893-2896.

HUANG, C.W., OKUBO, H. & UEMOTO, S. 1990a. Comparison of Bulblet formation from twin scales and single scales in *Hippeastrum hybridum* cultured *in vitro*. *Scientia Horticulturae* **42**: 151-160.

HUANG, C.W., OKUBO, H. & UEMOTO, S. 1990b. Importance of two scales in propagating *Hippeastrum hybridum* by twin scaling. *Scientia Horticulturae* **42**: 141-149.

HUANG, L.-C. & LIU, D.-M. 1989. Clonal multiplication of *Lycoris aurea* by tissue culture. *Scientia Horticulturae* **40**: 145-152.

HUGHES, K.W. 1981. Ornamental species. In: Conger, B.V. (Ed.). *Cloning Agricultural Plants via in vitro Techniques*. CRC Press, Boca Raton. ISBN 0-8493-5797-7.

HUSSEY, G. 1975a. Totipotency in tissue explants and callus of some members of the Liliaceae, Iridaceae, and Amaryllidaceae. *Journal of Experimental Botany* **26**: 253-262.

HUSSEY, G. 1975b. Propagation of hyacinths by tissue culture. *Scientia Horticulturae* **3**: 21-28.

HUSSEY, G. 1976. Propagation of Dutch Iris by tissue culture. *Scientia Horticulturae* **4**: 163-165.

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. & Gregory, M. (Eds.). *Petaloid Monocotyledons*. Academic Press, London. ISBN 0-12-133950-5.

HUSSEY, G. 1982. *In vitro* propagation of *Narcissus*. *Annals of Botany* **49**: 707-719.

HUSSEY, G. 1986. Vegetative Propagation of Plants by Tissue Culture. In: Yeoman, M.M. (Ed.). *Plant cell culture technology. Botanical Monographs Volume 23*. Blackwell Scientific Publications, Oxford. ISBN 0-632-01393-1.

HUSSEY, G. & FALAVIGNA, A. 1980. Origin and production of *in vitro* adventitious shoots in the Onion, *Allium cepa* L. *Journal of Experimental Botany* **31**: 1675-1686.

HUSSEY, G. & HILTON, J. 1977. *In vitro* propagation of *Narcissus*. *John Innes Annual Report* **68**: 45-46.

HUTCHINGS, A. & VAN STADEN, J. 1994. Plants used for stress-related ailments in traditional Zulu, Xhosa and Sotho medicine. Part 1: Plants used for headaches. *Journal of Ethnopharmacology* **43**: 89-124.

HUTCHINGS, A., SCOTT, A.H., LEWIS, G. & CUNNINGHAM, A. 1996. Zulu *Medicinal Plants: An Inventory*. University of Natal Press, Pietermaritzburg. ISBN 0-86980-923-7.

JACOBS, G., RICHARD, M., ALLDERMAN, L.A. & THERON, K.I. 1992. Direct and indirect organogenesis in tissue culture of *Nerine bowdenii* W. Watts. *Acta Horticulturae* **325**: 475-479.

JÄGER, A., MCALISTER, B.G. & VAN STADEN, J. 1995. *In vitro* culture of *Babiana* species. *Acta Horticulturae* **420**: 107-108.

JHA, S., MITRA, G.C. & SEN, S. 1984. *In vitro* regeneration from bulb explants of Indian squill, *Urginea indica* Kunth. *Plant Cell, Tissue and Organ Culture* **3**: 91-100.

KARIUKI, W. 2008. Rapid multiplication of *Ornithogalum saundersiae* Bak. through bulblet production *in vivo*. *Acta Horticulturae* **766**: 135-141.

KIM, K. & DE HERTOGH, A.A. 1997. Tissue culture of ornamental flowering bulbs (geophytes). *Horticultural Reviews* **18**: 87-169.

KOOPOWITZ, H. 1986. Conservation problems in the Amaryllidaceae. *Herbertia* **42**: 21-25.

KROMER, K. 1985. Regeneration of some monocotyledonous plants from subterranean organs *in vitro*. *Acta Agrobotanica* **38**: 65-87.

KROMER, K. 1989. The effect of light conditions on regeneration and level of endogenous growth regulators in *Muscari racemosum* (L) Mill. Bulb-scale sections cultured *in vitro*. *Acta Horticulturae* **251**: 173-181.

KUKUŁCZANKA, K. & KROMER, K. 1988. Propagation of *Vallota purpurea* Herb. through tissue culture. *Acta Horticulturae* **226**: 129-135.

KUKUŁCZANKA, K, KROMER, K. & CZASTKA, B. 1989. Propagation of *Fritillaria meleagris* L. through tissue culture. *Acta Horticulturae* **251**: 147-153.

KUTCHAN, T.M. 1995. Alkaloid Biosynthesis- The basis for metabolic engineering of medicinal plants. *The Plant Cell* **7**: 1059-1070.

LANGENS-GERRITS, M. & NASHIMOTO, S. 1997. Improved protocol for the propagation of *Narcissus in vitro*. *Acta Horticulturae* **430**: 311-313.

LEIFFERT, C. & WAITES, W.M. 1994. Dealing with microbial contaminants in plant tissue culture. Hazard analysis and critical control points. In: Lumsden. P.J., Nicholas, J.R. & Davies, W.J. (Eds.). *Physiology, Growth and Development of Plants in Culture*. Kluwer, Dordrecht. ISBN 0-7923-2516-8.

LESHEM, B., LILIEN-KIPNIS, H. & 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**: 129-260.

LIAN, M.-L., CHAKRABARTY, D. & PAEK, K.-Y. 2003. Growth of *Lilium* oriental hybrid 'Casablanca' bulblet using bioreactor culture. *Scientia Horticulturae* **97**: 41-48.

MAESATO, K., SHARADA, K., FUKUI, H., HARA, T. & SARMA, K.S. 1994. *In vitro* bulblet regeneration from bulbscale explants of *Lilium japonicum* Thumb. Effect of plant growth regulators and culture environment. *Journal of Horticultural Science* **69**: 289-297.

MANNING, J., GOLDBLATT, P. & SNIJMAN, D. 2002. *The Color Encyclopaedia of Cape Bulbs*. Timber Press, Portland. ISBN 0-8819-2547-0.

MCDONALD, M. 1985. Propagation of two kinds of South African bulbs. *Proceedings of the International Plant Propagation Society* **35**: 328-330.

MII, M., MORI, T. & IWASE, N. 1974. Organ formation from the excised bulb scales of *Hippeastrum hybridum in vitro*. *Journal of Horticultural Science* **49**: 241-244.

MOCHTAK, E. 1989. Bulblet formation from the explants of *Nerine bowdenii* W. Wats. *Acta Horticulturae* **251**: 199-204.

MORÁN, G.P., COLQUE, R., VILADOMAT, F., BASTIDA, J. & CODINA, C. 2003. Mass propagation of *Cyrtanthus clavatus* and *Cyrtanthus spiralis* using liquid medium culture. *Scientia Horticulturae* **98**: 49-60.

MURASHIGE, T. & SKOOG, F. 1962. A revised medium for rapid growth and bio assay with tobacco tissue cultures. *Physiologia Plantarum* **15**: 473-479.

NAYAK, S & SEN, S. 1995. Rapid and stable propagation of *Ornithogalum umbellatum* L. in long term culture. *Plant Cell Reports* **15**: 150-153.

NEUWINGER, H.D. 1996. *African Ethnobotany: Poisons and Drugs*. Chapman and Hall, Stuttgart. ISBN 3-8261-0077-8.

NEWTON, D.J. & 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.

NGUGI, G.W., JÄGER, A.K. & VAN STADEN, J. 1998. *In vitro* propagation of *Drimia robusta* Bak. *South African Journal of Botany* **64**: 266-268.

NHUT, D.T. 1998. Micropropagation of lily (*Lilium longiflorum*) via *in vitro* stem node and pseudo-bulblet culture. *Plant Cell Reports* **17**: 913-916.

NIEDERWIESER, J.G., TERBLANCHE, M. & SPREETH, M.H. 2002. Potential of South African members of the Amaryllidaceae for new crop development. *Acta Horticulturae* **570**: 359-365.

NIELSEN, N.D., SANDAGER, M., STAFFORD, G.I., VAN STADEN, J. & JÄGER, A.K. 2004. Screening of indigenous plants from South Africa for affinity to the serotonin reuptake transport protein. *Journal of Ethnopharmacology* **94**: 159-163.

NIIMI, Y., MISAKI, Y. & NAKANO, M. 2000. Production of commercial bulbs of *Lilium rubellum* Baker: changes in carbohydrates in bulblets and sugars of liquid medium during their culture. *Journal of Japanese Society Horticultural Science* **69**: 161-165.

OKUBO, H., HUANG, C.W. & 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**: 59-66.

OLIVER, I.B. 1990. *Crinum bulbispermum* - the flower of the Orange Free State. *Veld & Flora* June: 57.

OLIVIER, W. 1983. Rare Amaryllidaceae from Southern Africa. *Veld & Flora* December: 162-165.

ORAN, S.A. & FATTASH, I.A. 2005. *In vitro* propagation of an endangered medicinal bulbous plant *Sternbergia clusiana* Ker-Gawler (*Amaryllidaceae*). *Journal of Horticultural Science & Biotechnology* **80**: 399-402.

OZEL, C.A., KHAWAR, K.M., KARAMAN, S., ATES, M.A. & ARSLAN, O. 2008. Efficient *in vitro* multiplication in *Ornithogalum ulophyllum* Hand.-Mazz. from twin scale explants. *Scientia Horticulturae* **116**: 109-112.

PAEK, K.Y. & MURTHY, H.N. 2002. High frequency of bulblet regeneration from bulb scale sections of *Fritillaria thunbergii*. *Plant Cell, Tissue and Organ Culture* **68**: 247-252.

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

PECK, D.E. & 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.

PIENAAR, K. 1994. *The Ultimate Southern African Gardening Book*. Southern Book Publishers, Halfway House. ISBN 1868125505.

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.

PIERIK, R.L. M. & IPPEL, B.J. 1977. Plantlet formation from excised bulb scale segments of *Nerine*. *Acta Horticulturae* **78**: 197-202.

PIERIK, R.L.M. & RUBING, 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., SPRENKELS, P.A. & VAN BRAGT, J. 1983. Rapid vegetative propagation of *Eucharis grandiflora in vitro*. *Acta Horticulturae* **147**: 179-186.

POOLEY, E. 1998. *A Field Guide to Wild Flowers of KwaZulu-Natal and the Eastern Region*. Natal Flora Publications Trust, Durban. ISBN 0-620-21500-3.

PRASAD, V.S.S. & DUTTA GUPTA, S. 2006. *In vitro* shoot regeneration of gladiolus in semi-solid agar versus liquid cultures with support systems. *Plant Cell, Tissue and Organ Culture* **87**: 263-271.

RAMOGOLA, W.P.N. & FENNELL, C.W. 2007. The use of inflorescence explants for micropropagating *Eucomis zambesiaca*. *South African Journal of Botany* **73**: 334.

READ, M. 1989. Over exploitation of wild bulbs by the horticultural trade. *Herbertia* **45**: 6-12.

REMOTTI, P.C. & LÖFFLER, J.M. 1995. Callus induction and plant regeneration from gladiolus. *Plant Cell, Tissue and Organ Culture* **42**: 171-178.

ROBB, S.H. 1957. The culture of excised tissue from bulb scales of *Lilium speciosum*. *Journal of Experimental Botany* **8**: 348-352.

SANTOS, A., FIDALGO, F., SANTOS, I. & SALEMA, R. 2002. *In vitro* bulb formation of *Narcissus asturiensis*, a threatened species of the Amaryllidaceae. *Journal of Horticultural Science & Biotechnology* **77**: 149-152.

SANTOS, J., SANTOS, I. & SALEMA, R. 1998. *In vitro* production of bulbs of *Narcissus bulbocodium* flowering in the first season of growth. *Scientia Horticulturae* **76**: 205-217.

SCOTT, S.J., JONES, R.A. & WILLIAMS, W.A. 1984. Review of data analysis methods for seed germination. *Crop Science* **24**: 1192-1199.

SEABROOK, J.E.A. & CUMMING, B.G. 1982. *In vitro* morphogenesis and growth of *Narcissus* in response to temperature. *Scientia Horticulturae* **16**: 185-190.

SELLÉS, M., BERGOÑÓN, S., VILADOMAT, F., BASTIDA, J. & CODINA, C. 1997. Effect of sucrose on growth and galanthamine production in shoot-clump cultures of *Narcissus confuses* in liquid-shake medium. *Plant Cell, Tissue and Organ Culture* **49**: 129-136.

SELVARAJ, N. ,VASUDEVAN, A., MANICKAVASAGAM, M., KASTHURIRENGAN, S. & GANAPATHI, A. 2007. High frequency shoot regeneration from cotyledon explants of cucumber via organogenesis. *Scientia Horticulturae* **112**: 2-8.

SEN, J. & SHARMA, A.K. 1991. Micropropagation of *Withania somnifera* from germinating seeds and shoot tips. *Plant Cell, Tissue and Organ Culture* **26**: 71-73.

SHUSHU, D.D., MUTANYATTA, J. & ABEGAZ, B.M. 2005. *In vitro* propagation and phytochemical investigation of *Ledebouria graminifolia*, an important medicinal bulb in southern Africa. *Journal of Herbs, Spices & Medicinal Plants* **11**: 97-107.

SLABBERT, M.M., DE BRUYN, M.H., FERREIRA, D.I. & PRETORIUS, J. 1993. Regeneration of bulblets from twin scales of *Crinum macowanii in vitro*. *Plant Cell, Tissue and Organ Culture* **33**: 133-141.

SNIJMAN D.A. & ARCHER, R.H. 2003. Amaryllidaceae. In: Germishuizen, G. & Meyer, N.L. (Eds.). Plants of Southern Africa: an annotated checklist. *Strelitzia* **14**: 957-967. National Botanical Institute, Pretoria.

SQUIRES, W.M., LANGTON, F.A. & FENLON, J.S. 1991. Factors influencing the transplantation success of micropropagated *Narcissus* bulbils. *Journal of Horticultural Science* **66**: 661-671.

STANCATO, G.C., MAZZAFERA, P. & MAGALHÃES, A.C. 1995. Dry matter partitioning during the propagation of *Hippeastrum hybridum* as affected by light. *Scientia Horticulturae* **62**: 81-87.

STEINITZ, B. & YAHEL, H. 1982. *In vitro* propagation of *Narcissus tazetta*. *HortScience* **17**: 333-334.

STIRTON, C.H. 1980. Aspects of research on South Afican petaloid monocotyledons of horticultural importance. In: Brickwell, C.D., Cutler, D.F. & Gregory, M. (Eds.). *Petaloid Monocotyledons*. Academic Press, London. ISBN 0-12-133950-5.

SWEET, H.C. & BOLTON, W.E. 1979. The surface decontamination of seeds to produce axenic seedlings. *American Journal of Botany* **6**: 692-698.

TAEB, A.G. & 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**: 193-197.

TAKAYAMA, S. & MISAWA, M. 1979. Differentiation in *Lilium* bulbscales grown *in vitro*. Effect of various cultural conditions. *Physiologia Plantarum* **46**: 184-190.

TAKAYAMA, S. & 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. & MISAWA, M. 1983. A scheme for mass propagation of *Lilium in vitro*. *Scientia Horticulturae* **18**: 353-362.

THOMPSON, P. 1989. *Creative Propagation: A Growers Guide*. Christopher Helm, London. ISBN 0-7470-3213-0.

ULRICH, M.R., DAVIES, F.T. JR., KOH, Y.C., DURAY, S.A., EGILLA, J.N. 1999. Micropropagation of *Crinum* 'Ellen Bosanquet' by tri-scales. *Scientia Horticulturae* **82**: 95-102.

VAN AARTRIJK, J. & 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. & VAN DER LINDE, P.C.G. 1986. *In vitro* propagation of flower bulb crops. In: Zimmerman, R.H., Hammerschlag, F.A. & 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. 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., BLOM-BARNHOORN, G.J. & VAN AARTRIJK, J. 1986. Towards "in vitro" propagation of bulbous Iris. *Acta Horticulturae* **177**: 569.

VAN DER LINDE, P.C.G., HOL, G.M.G.M., BLOM-BARNHOORN, G.J., VAN AARTRIJK, J. & 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 RENSBURG, J.G.J., VCELAR, B.M. & LANDBY, P.A. 1988. Micropropagation of *Ornithogalum maculatum*. *South African Journal of Botany* **55**: 137-139.

VAN STADEN, J., ELMER-ENGLISH, C.W. & FINNIE, J.F. 1991. Physiological and anatomical aspects related to *in vitro* shoot initiation in *Bowiea volubilis*. *South African Journal of Botany* **57**: 352-355.

VAN STADEN, J., ZAZIMALOVA, E. & GEORGE, E.F. 2008. Plant growth regulators II: Cytokinins, their analogues and antagonists. In: George, E.F., Hall, M.A. De Klerk, G.-J. *Plant Propagation by Tissue Culture 3rd edition, Vol. 1. The background*. Springer, Dordrecht. ISBN 9781402050053.

VAN WYK, B.-E. & GERICKE, N. 2000. *People's Plants: A Guide to Useful Plants of Southern Africa*. Briza Publications, Pretoria. ISBN 1-8750-9319-2.

VARSHNEY, A., DHAWAN, V. & SRIVASTAVA, P. 2000. A protocol for *in vitro* mass propagation of Asiatic hybrids of Lily through liquid stationary culture. *In Vitro Cellular Developmental Biology* **36**: 383-391.

VERDOORN, I.C. 1973. The genus *Crinum* in southern Africa. *Bothalia* **11**: 27-52.

VILADOMAT, F., ALMANZA, G.R., CODINA, C., BASTIDA, J., CAMPBELL, W.E. & MATHEE, S. 1996. Alkaloids from *Brunsvigia orientalis. Phytochemistry* **43**: 1379-1384.

VILADOMAT, F., BASTIDA, J., CODINA, C., CAMPBELL, W.E. & MATHEE, S. 1995a. Alkaloids from *Boophane flava*. *Phytochemistry* **40**: 307-311.

VILADOMAT, F., CODINA, C., BASTIDA, J., MATHEE, S. & CAMPBELL, W.E. 1995b. Further alkaloids from *Brunsvigia josephinae*. *Phytochemistry* **40**: 961-965.

WANG, P.-J. & HUANG, L.-C. 1976. Beneficial effects of activated charcoal on plant tissue and organ cultures. *In Vitro* **12**: 260-262.

WATAD, A.A., KOCHBA, M., NISSIM, A. & GABA, V. 1995. Improvement of *Aconitum napellus* micropropagation by liquid culture on floating membrane rafts. *Plant Cell Reports* **14**: 345-348.

WATT, J.M. & BREYER-BRANDWIJK, M.G. 1962. *The Medicinal and Poisonous Plants of Southern and Eastern Africa*. *Second Edition*. E. & S. Livingstone Ltd., Edinburgh.

WEATHERHEAD, M.A., BURDON, J. & HENSHAW, G.G. 1978. Some effects of activated charcoal as an additive to plant tissue culture media. *Zeitscrift fűr Pflanzenphysiologie* **89**: 141-147.

WITOMSKA, M. & LUKASZEWSKA, A.J. 1997. Bulblet regeneration *in vitro* from different explants of *Fritillaria imperialis*. *Acta Horticulturae* **430**: 331-338.

YANAGAWA, T. & SAKANISHI, N. 1980a. Studies on the regeneration of excised bulb tissues of various tunicated-bulbous ornamentals, I: regenerative capacity of the segments from different parts of bulb scales. *Journal of the Japanese Society of Horticultural Science* **48**: 495-502.

YANAGAWA, T. & SAKANISHI, N. 1980b. Studies on the regeneration of excised bulb tissues of various tunicated-bulbous ornamentals, II: morphological observations on bulblet formation from bulb-scale segments. *Journal of the Japanese Society of Horticultural Science* **49**: 119-126.

YEOMAN, M.M. 1986. *Plant Cell Culture Technology. Botanical monographs Volume 23*. Blackwell Scientific Publications, Oxford. ISBN 0-632-01393-1.

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., HERTZ, N. & BIRAN, Y. 1983. Vegetative reproduction of *Allium ampeloprasum* L. *in vivo* and *in vitro*. *Israel Journal of Botany* **32**: 1-9.

ZIV, M., KAHANY, S. & LILIEN-KIPNIS, H. 1995. Somatic embryos and bulblet development from bioreactor regenerated meristematic clusters of *Nerine*. *Acta Horticulturae* **393**: 203-212.

ZIV, M. & LILIEN-KIPNIS, H. 1997. The inflorescence stalk: A source of highly regenerative explants for micropropagation of geophytes. *Acta Horticulturae* **447**: 107-109.

ZIV, M. & LILIEN-KIPNIS, H. 2000. Bud regeneration from inflorescence explants for rapid propagation of geophytes *in vitro*. *Plant Cell Reports* **19**: 845-850.

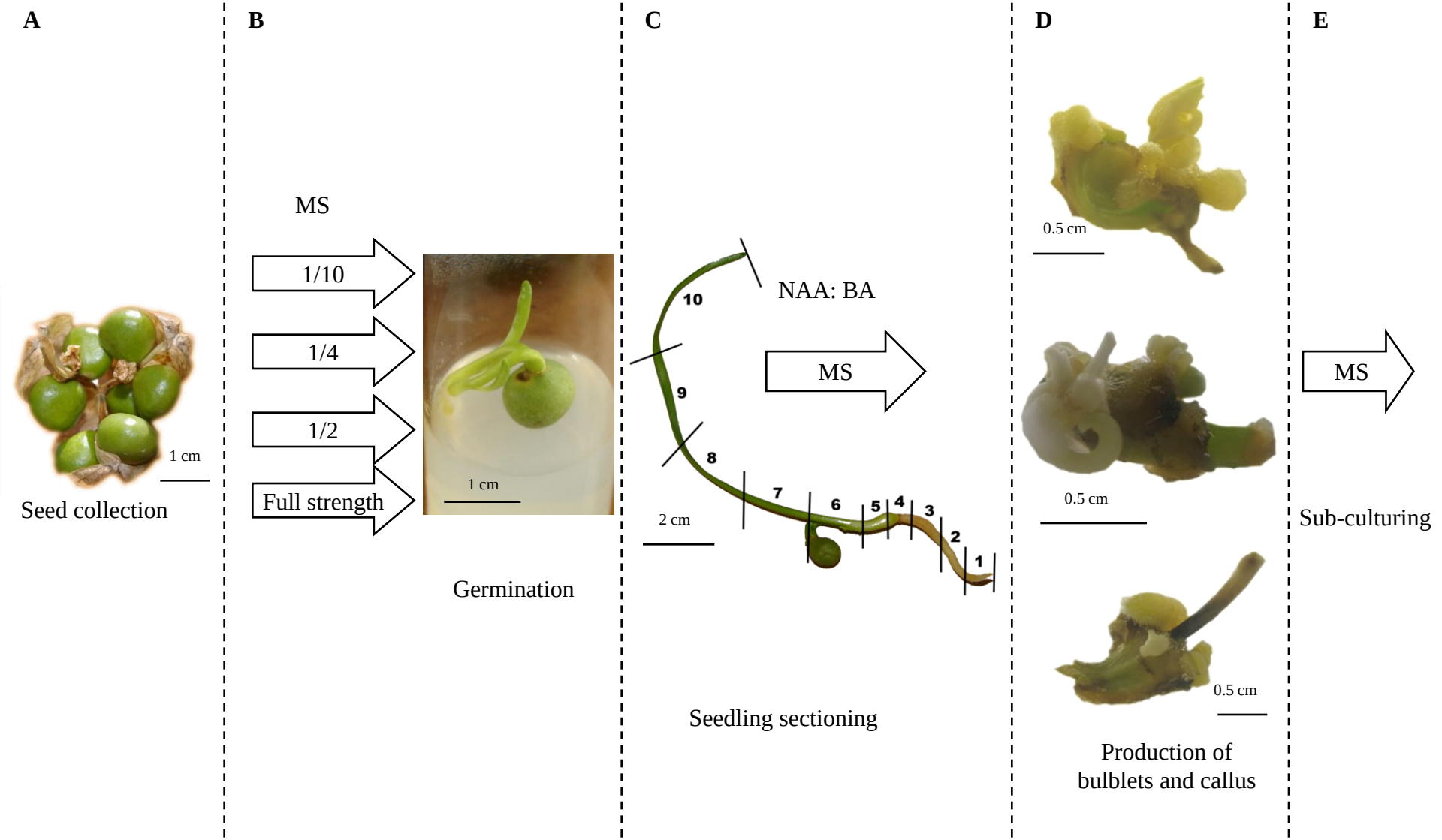


Figure 2.1 (A-E): An overview of the methods used in the tissue culture of *B. undulata* from seeds.

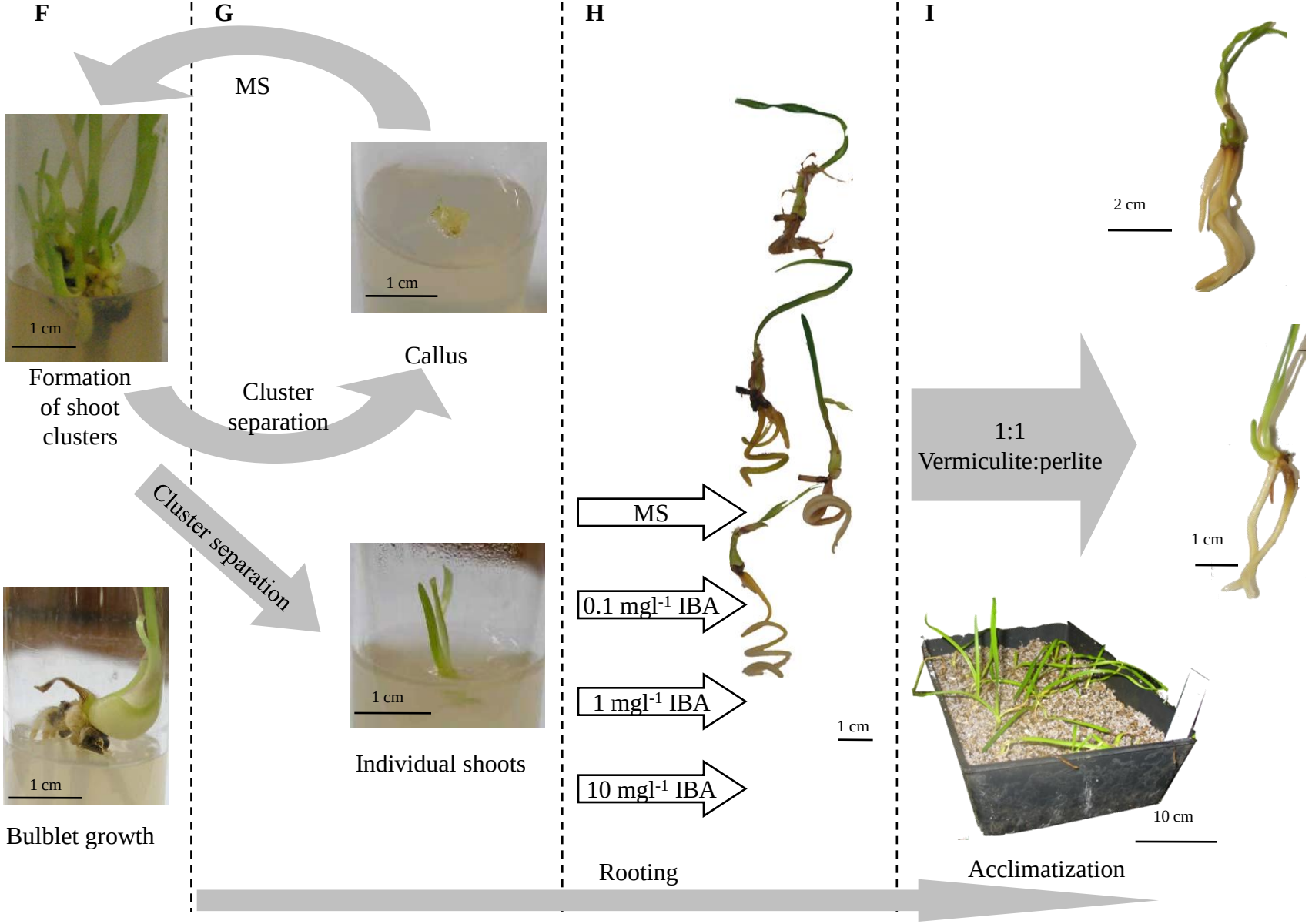


Figure 2.1 (F-I): An overview of the methods used in the tissue culture of *B. undulata* from seeds.

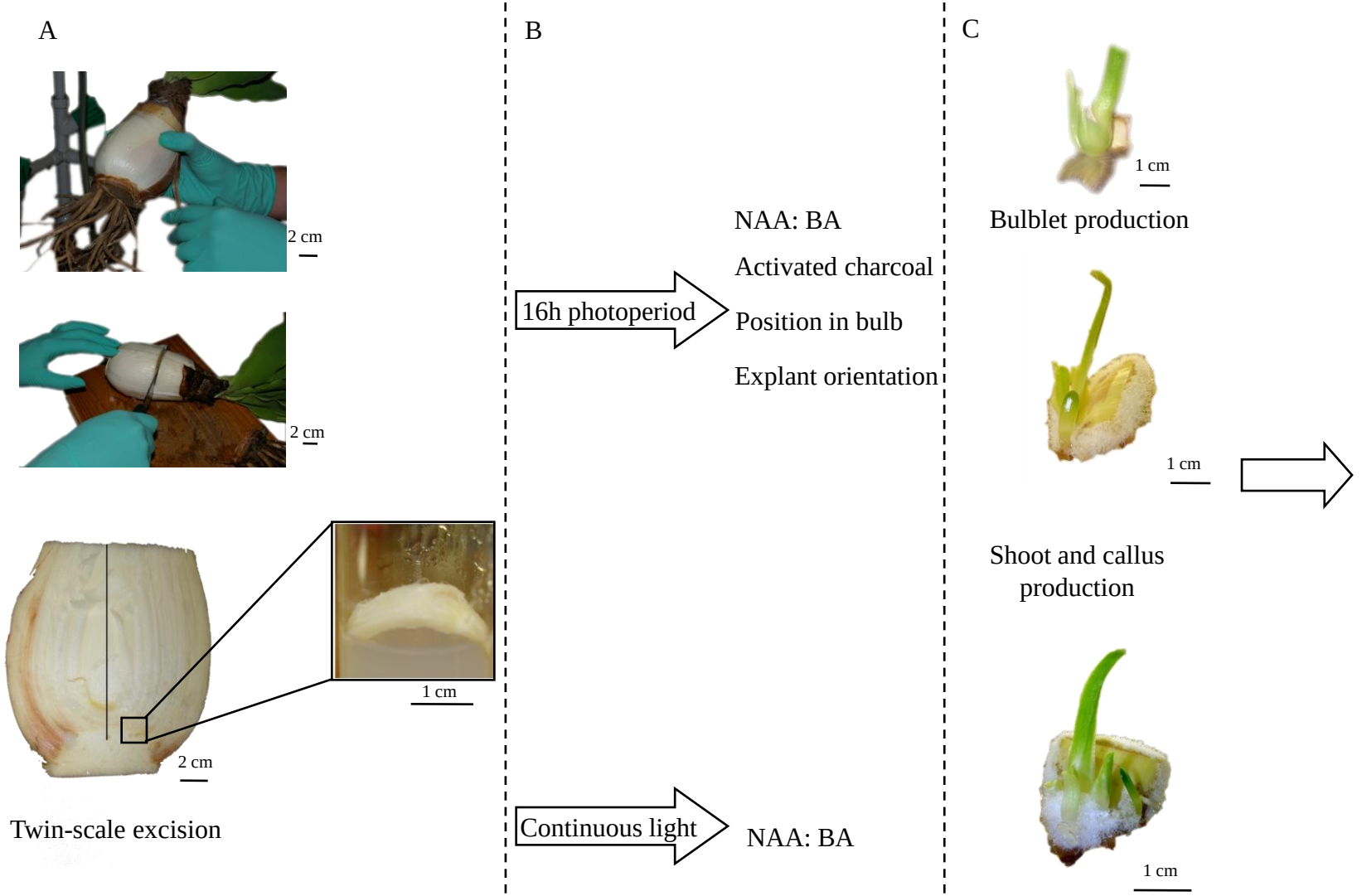


Figure 3.1 (A-C): An overview of the methods used in the tissue culture of *B. undulata* from twin-scales.

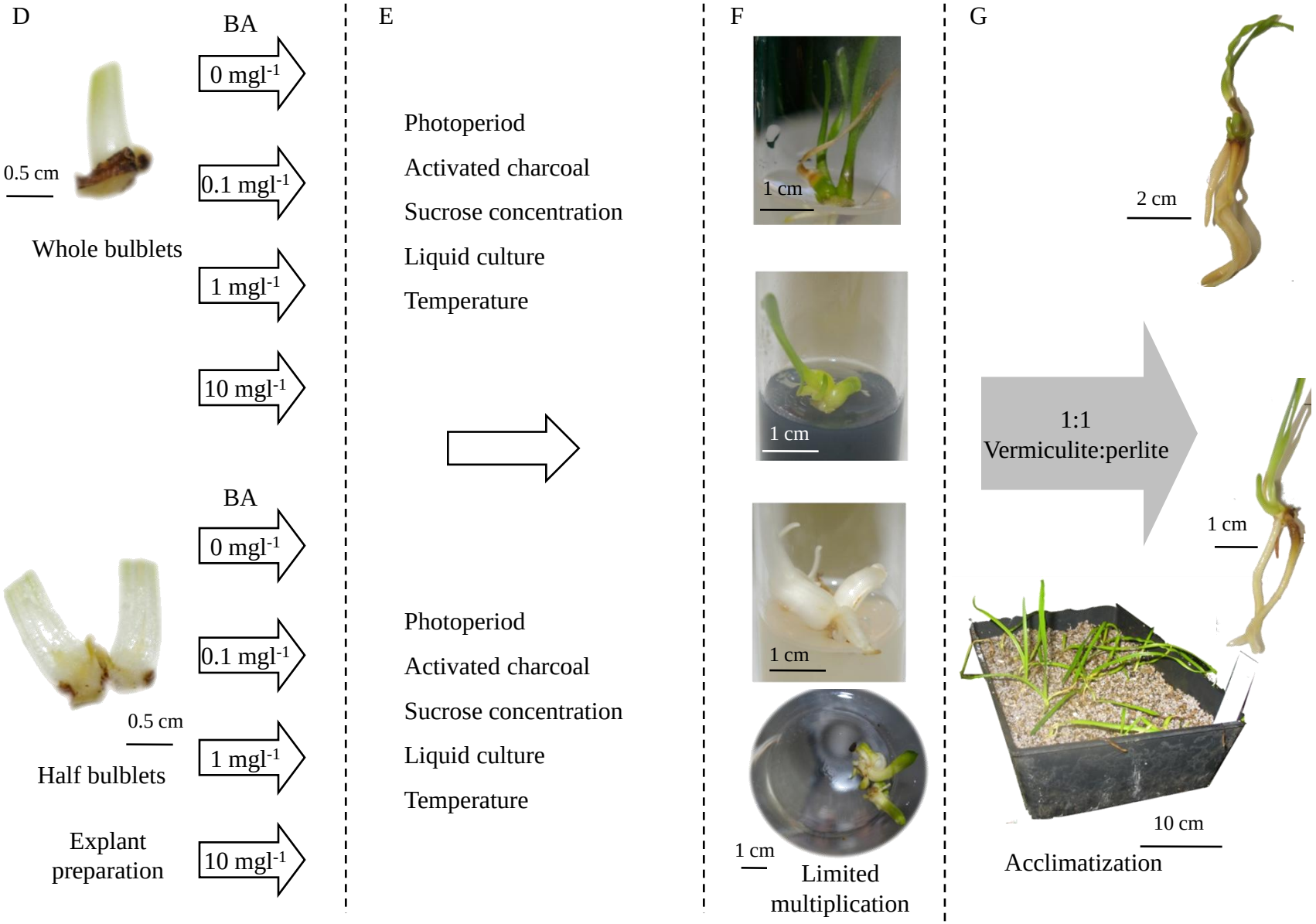


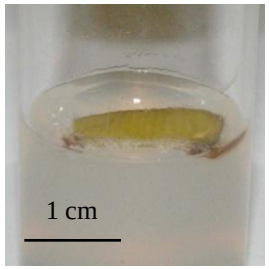
Figure 3.1 (D-G): An overview of the methods used in the tissue culture of *B. undulata* from twin-scales.

Leaf collection

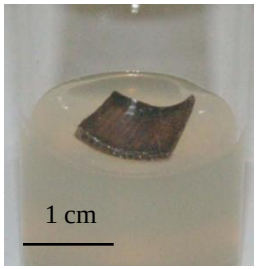
NAA:BA

Abaxial

Adaxial



Callus



Browning

Flower collection

MS

No organogenesis

Figure 3.23: An overview of the methods used in the tissue culture of *B. undulata* from leaf and flower explants.