In vitro bulb induction in *Eucomis zambesiaca* Baker.

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Masters of Science

Research Centre for Plant Growth and Development
School of Biological and Conservation Sciences
University of KwaZulu-Natal, Pietermaritzburg
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Student Declaration

In vitro bulb induction in *Eucomis zambesiaca* Baker.

I, Lee Cheesman, Student Number 203502173

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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. J.F. Finnie

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Abstract

Eucomis L' Hér. is a genus of 10 species that fall within the Hyacinthaceae family. *Eucomis zambesiaca* Baker is a summer-blooming bulbous geophyte occurring from northern South Africa to Malawi. *Eucomis* species are used in southern African traditional medicine for the treatment of various ailments, in particular, pain and inflammation. As a result, the bulbs are heavily harvested for trade in South Africa's traditional 'muthi' markets. Over-collection of *Eucomis* species has seriously depleted natural populations and now *Eucomis* plants are among the 15 scarcest medicinal species to be traded.

Micropropagation is a useful technique for rapid clonal multiplication of plant material which could potentially yield useful secondary metabolites as well as alleviate the pressure on the wild plant populations. The *in vitro* induction of storage organs is especially beneficial as it can limit the loss of plants during acclimatization as bulblets are hardier than shoots or plantlets. The aim of this research was to determine optimal growth conditions for bulblet induction of *Eucomis zambesiaca*.

The effect of environmental and physiological parameters on the initiation and growth of bulblets was investigated. These included the effect of temperature, photoperiod, various carbohydrates at different concentrations and combinations as well as various plant growth regulators. Maximum number of bulblets per explant was obtained at 20 ℃, with an average of three bulbs per leaf explant. The average bulblet mass was 57 mg, which was significantly higher than bulblets formed at other tested temperatures. An 8 h light regime was the optimum photoperiod. The highest mean number of bulblets (1.4 per leaf explant) developed under the 8 h photoperiod and the bulblets that formed were large in size. They had a mean bulb diameter of 3.4 mm and a mean bulb weight of 42 mg.

Different carbohydrates such as fructose, sucrose and glucose were tested at concentrations of; 1, 3, 6, 9 and 12%. Fructose at a concentration of 3% was found to

produce the best results. An average of 1.2 bulbs formed per explant. The mean bulb diameter was 3.4 mm and mean bulb weight was 56.6 mg.

Plant growth regulators (GA_3, IAA, IBA, NAA, BA, zeatin, iP and others) were tested at concentrations of 1, 2 and 5 mg/L. 1 mg/L IBA was found to be the optimum hormone treatment for bulblet induction. Bulblets were large, had good leaves and well established roots. Medium supplemented with 1 mg/L IBA produced bulblets that had an average bulb diameter of 4.36 mm and a mean bulblet weight of 79.1 mg.

Bulblets grown *in vitro* were transferred to vermiculite and placed in a misthouse to acclimatize. After 2 months the plantlets were transferred to pots containing a sand:soil mixture of 1:1 and placed in a greenhouse. There was a 80 to 90% survival rate.

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List of Abbreviations

ABA.....	abscisic acid
BA	N^6-benzyladenine; 6-benzylaminopurine
[3G]BA	3-glycosylbenzyladenine
[7G]BA	7-glycosylbenzyladenine
[9G]BA	9-glycosylbenzyladenine
2,4-D	2,4-dichlorophenoxy acetic acid
ft.-c.	foot-candle (for cool white fluorescent lamps, 1 ft.-c. is approximately 0.146 µmol/m^2/s)
GAs.....	gibberellins
GA_3	gibberellic acid
GA_{4+7}.....	GA_4 and GA_7 gibberellin mixture
IAA	indole-3-acetic acid
IBA	indole-3-butyric acid
iP.....	N^6-isopentenyladenine
MeJa	methyl jasmonate
MS	Murashige and Skoog (1962) medium
*m*T.....	*meta*-topolin
NAA	napthaleneacetic acid
PAA.....	phenylacetic acid
PAC	paclobutrazol
PGR	plant growth regulator
rpm.....	revolutions per minute

Chapter 1
INTRODUCTION

South Africa has an extremely rich plant diversity and hosts approximately 30 000 plant species of which approximately 2700 are geophytes (FERREIRA & HANCKE, 1985; LOUW *et al.*, 2002; NIEDERWIESER *et al*., 2002). Some of these species have been grown for ornamental and culinary purposes or are used as medicinal plants.

Hyacinthaceae, which was formerly part of the Liliaceae, is a family that is made up of about 41 genera and approximately 770 species of deciduous bulbous plants (POOLEY, 2005). The family is well represented in South Africa. Over half the genera (27) are found in southern Africa and almost 200 species are from the Cape Floristic Region alone. The genus *Eucomis* L' Hérit belongs to the family Hyacinthaceae and is classified in the order Asparagales and the class Liliopsida (DAHLGREN *et al.*, 1985; COMPTON, 1990). The genus comprises of about 10 species (POOLEY, 2005).

The indigenous bulbous plants that are of importance to traditional healers, mainly belong to the Amaryllidaceae and Hyacinthaceae families (LOUW et al., 2002). Members of the Hyacinthaceae are highly valued for their medicinal properties and according to AFOLAYAN and ADEBOLA (2004), they make up about 14% of the medicinal plants traded in South Africa.

The bulbs of *Eucomis* species are greatly valued in traditional medicine for the treatment of various ailments (WATT & BREYER-BRANDWIJK, 1962; TAYLOR & VAN STADEN, 2001c; AFOLAYAN & ADEBOLA, 2004). As a result, the bulbs are heavily harvested for trade in South Africa's traditional 'muthi' markets. Over-collection of *Eucomis* species has seriously depleted natural populations and now *Eucomis* plants are among the 15 scarcest medicinal species traded. This is

confirmed by both urban and rural herb traders and herbalists in South Africa (CUNNINGHAM, 1988). Other factors contributing to the vulnerability of *Eucomis* species include low population size and slow growth rates (DE LANGE *et al.*, 1989). Although the conservation status of *Eucomis* is described as declining, certain species such as *E. bicolor* are classified as threatened (SCOTT-SHAW, 1999). Therefore, the problem of over-exploitation has to be addressed.

According to NIEDERWIESER *et al*. (1998), in the ten year period between 1988 and 1998, the value of the flower industry increased in South Africa and currently has an approximate value in excess of R426 million. In general the demand for flower bulbs has also increased in the ten year period along with the commercial value doubling (NIEDERWIESER *et al*., 1998). *Eucomis* species show great potential for the cut flower industry, as the flowers are attractive and long lasting, as well as for the sale of dry bulbs for use in gardens and as pot plants (TAYLOR & VAN STADEN, 2001c). According to TAYLOR and VAN STADEN (2001c) several of the smaller *Eucomis* species such as *E. zambesiaca* and *E. humilis* show great horticultural potential as pot plants and the larger species would make great garden plants. Thus *Eucomis* species have potential to become commercially important plants.

Therefore, the aim of this research was to develop a protocol that optimizes the production of bulblets *in vitro*. This protocol will hopefully be valuable to the horticultural industry as well as a way to produce bulbs that can be used by traditional healers and in so doing, prevent large scale collection from the wild.

1.1 DISTRIBUTION AND MORPHOLOGY

Eucomis species are ornamental geophytes with a wide distribution. Most of the species occur in the eastern and north-eastern areas of South Africa, however, *Eucomis regia* (L.) L' Hér. is found in the western and south-western Cape while *Eucomis zambesiaca* Baker. is found from northern South Africa to Malawi (PIENAAR, 1984; DU PLESSIS & DUNCAN, 1989). All the species are summer-

blooming and dormant in winter, except for *E. regia* which is winter-growing and dies down in summer.

Eucomis species have a large, ovoid or globose bulb that is covered with a hard, shiny, tunic of membranous leaf bases (BRYAN, 1989; FABIAN & GERMISHUIZEN, 1997). The bulbs produce a rosette of long, smooth, basal leaves that vary in shape from lanceolate or elliptic to ovate (PIENAAR, 1985; BRYAN, 1989; DU PLESSIS & DUNCAN, 1989; FABIAN & GERMISHUIZEN, 1997). The margins are wavy. The leaves arch and often curve back to the ground, resting there, which makes it look a little untidy (BRYAN, 1989).

Eucomis, which comes from the Greek word *eukomes*, meaning 'beautifully haired', refers to the tuft of leaf-like bracts at the top of the flower spike (PIENAAR, 1985; BRYAN, 1989; DU PLESSIS & DUNCAN, 1989). It is this unusual structure of the inflorescence that makes *Eucomis* species popular ornamentals. The bulb produces an erect, cylindrical inflorescence. The stem varies in height with species from 30 cm to 100 cm. The pale lime-green flower spike or raceme is densely packed with white, pale green, purple-tinted or yellowish-green flowers, depending on the species. The flowers may be sweetly or unpleasantly scented (BRYAN, 1989; DU PLESSIS & DUNCAN, 1989; FABIAN & GERMISHUIZEN, 1997).

Image from www.diggingdog.com

Once the flowers have faded, attractive seed pods follow. The swollen fruits are either green or brown and are almost triangular in shape. Within the fruit are hard, round, black or brown seeds (PIENAAR, 1985; BRYAN, 1989; DU PLESSIS & DUNCAN, 1989).

1.2 HORTICULTURAL USE

All *Eucomis* species are highly ornamental due to the unusual eye-catching 'pineapple' inflorescence. Due to this unusual structure it is frequently referred to as the 'pineapple' plant. The plants can be grown in the garden, rockery or planted in containers on a sunny veranda. The flowers are particularly long-lasting and so, can be used as cut flowers (DU PLESSIS & DUNCAN, 1989).

1.3 MEDICINAL AND OTHER USES

1.3.1 Traditional medicinal uses

There is little information available regarding the specific medicinal use of *Eucomis zambesiaca*. The bulbs of other *Eucomis* species, however, are highly valued and often used in Zulu, Xhosa and Tswana traditional medicine owing to their anti-inflammatory properties (WATT & BREYER-BRANDWIJK, 1962; HUTCHINGS, 1989, HUTCHINGS, 1996). The bulbs of *Eucomis autumnalis* subsp *autumnalis* and *Eucomis autumnalis* subsp *clavata* are used in decoctions which are administered as enemas. These are used to treat lower backache, biliousness, urinary diseases, to help in post-operative recovery, for fevers as well as to assist in the healing of fractures (HUTCHINGS, 1996; VAN WYK *et al.,* 1997).

Decoctions made by boiling the bulb and roots of *Eucomis bicolor* and *Eucomis autumnalis* subsp *autumnalis* in milk or water are taken for colic, flatulence and for the relief of abdominal distension by the Tswana. The Zulu take a decoction of the bulb of a *Eucomis* sp. (probably *Eucomis regia*) for the treatment of coughs and other respiratory ailments (WATT & BREYER-BRANDWIJK, 1962; HUTCHINGS, 1996; VAN WYK *et al.,* 1997). The Southern Sotho use the root of *Eucomis regia* to stop diarrhoea and to prevent premature childbirth. Lastly, the Xhosa use a bulb decoction

of *Eucomis comosa* var *comosa* to treat rheumatism and it is also given as an enema to children during teething (WATT & BREYER-BRANDWIJK, 1962).

1.3.2 Other uses

The Tswana and Sotho use bulb decoctions of *Eucomis autumnalis* subsp *autumnalis* to treat hangovers and unspecified plant parts are used for syphilis and blood disorders (HUTCHINGS, 1996). The bulb of *Eucomis pole-evansii* is used by the African people in the Ventersdorp region as a medicine for people who suffer from mental diseases (WATT & BREYER-BRANDWIJK, 1962).

According to WATT and BREYER-BRANDWIJK (1962), the bulb of *Eucomis* sp. are also used, by both the Tswana and Southern Sotho, for magical purposes such as acting as a protective charm against witchcraft. It is believed that it is useful in inflicting harm on one's enemy.

The Southern Sotho use the plant as a remedy for venereal diseases of domestic stock (WATT & BREYER-BRANDWIJK, 1962; HUTCHINGS, 1996).

1.3.3 Isolated compounds

Antibiotics lose their effectiveness as they are used over time and resistant strains of bacteria develop. Thus there is an urgent need to identify novel active compounds for drug development. Hyacinthaceous plants have been used in Xhosa and Zulu remedies for many years to treat a number of ailments.

A large number of homoisoflavanones have been isolated from several hyacinthaceous genera including *Eucomis, Merwilla, Ledebouria, Veltheimia* and *Drimiopsis*. Homoisoflavanones belong to a small homogenous group of naturally occurring oxygen heterocycles. It has been reported that the biological activity of homoisoflavanones include anti-inflammatory, antibacterial, antihistaminic,

antimutagenic and angioprotective properties and has potent phosphodiesterase inhibitors (DU TOIT *et al.,* 2007).

Eucomnalin, eucomin, punctatin, autumnalin and 3,9-dihydroeucomnalin are some of the homoisoflavanones isolated from *Eucomis autumnalis.* Other compounds from *E. autumnalis* include the benzopyrones autumnariol and autumnariniol as well as some steroidal triterpenoides such as eucosterol (HUTCHINGS, 1996; VAN WYK *et al.,* 1997). Flavonoides are known for their anti-inflammatory and antispasmodic action, therefore, the beneficial effects of *Eucomis* extracts could be due to the homoisoflavanones, while the triterpenoides are beneficial in wound healing (VAN WYK *et al.,* 1997).

Much attention has been given to the pharmacological effects of natural peptides and proteins including lectins. GAIDAMASHVILI and VAN STADEN (2006) therefore examined lectin-like proteins from eight South African medicinal plant species for anti-inflammatory activity. *Eucomis autumnalis* was one of the eight species to be tested. It was found that *E. autumnalis* lectin solution showed the highest cyclooxygenase (COX) inhibitory activity compared with the other species tested. It is believed that the use of lectins does not produce the side effects associated with the use of nonsteroidal anti-inflammatory drugs and so these results show that there is much promise in the use of lectins in clinical-medicinal application.

JÄGER *et al.* (1996) screened aqueous and ethanolic extracts from 39 plants used in traditional Zulu medicine to treat headache or anti-inflammatory diseases. The COX assay was used to screen plant extracts and it was found that the highest inhibition was obtained using ethanolic extracts and *Eucomis autumnalis* gave some of the best results. These results are in agreement with TAYLOR and VAN STADEN (2001b) who screened extracts from all the *Eucomis* species for anti-inflammatory activity. It was found that the anti-inflammatory activity of ethanolic bulb extracts from all the species was high, while 9 of the 10 water extracts showed moderate anti-inflammatory activity. In general the highest anti-inflammatory activity was observed

in the bulbs and roots of the plants (JÄGER *et al.,* 1996; TAYLOR & VAN STADEN, 2001b).

As high levels of anti-inflammatory activity were found in *in vitro* plantlets of *E. autumnalis* subsp *autumnalis,* TAYLOR and VAN STADEN (2001a) looked at the production of these compounds by *in vitro* callus cultures. The anti-inflammatory activity of an extract prepared from callus was quantified by using the cyclooxygenase-1 and -2 assays. It was found that *E. autumnalis* callus exhibited more COX-2 inhibitory activity compared to COX-1.

Previous studies on *Eucomis* species have shown high levels of COX-1 inhibitory activity in ethanol extracts from leaves, bulbs and roots. In another investigation carried out by TAYLOR and VAN STADEN (2001f) a study of the COX-2 inhibitory activity from various plant parts of *E. autumnalis* subsp *autumnalis* showed that the crude extracts from the leaves and roots have significantly higher activity than from the bulb extracts. It was also found by TAYLOR and VAN STADEN (2001d) that young *Eucomis* plants have large amounts of COX-1 inhibitory activity especially in the leaves. As the plant matured, higher levels of activity were found in the roots and bulbs. However, there is actually no significant difference between the COX-1 inhibitory activity of the leaves, roots and bulbs. This suggests that the leaves could be used as a viable and sustainable alternative to the more destructive use of bulbs and roots. This is an important finding in terms of the conservation status of *Eucomis* species.

These screening procedures validated the extensive use of *Eucomis* species in southern African traditional medicine to treat many ailments.

1.4 PROPAGATION

1.4.1 Conventional propagation

The bulbs of *Eucomis* species must be planted in full sun or partial shade. The bulbs are planted 5 cm deep or with their tops at, or just below, the soil surface. They grow well in rich, well-drained soil. The bulbs should be allowed to remain in the ground for several seasons, allowing them to become well established. The plants do not like being lifted, so only lift when overcrowded (PIENAAR, 1985; BRYAN, 1989; DU PLESSIS & DUNCAN, 1989).

Eucomis species can be propagated from offsets or seeds, however, it takes three or more years to reach flowering age from seeds. Seed propagation is generally more promising and cost-effective for mass propagation of seedlings. There is, however, very little scientific information available about the seed biology of *Eucomis* species and only 65% of the seeds germinate with proper management (KULKARNI *et al.,* 2006). Therefore, the best method for the propagation of *Eucomis* plants is by offsets but even the offsets develop rather slowly (PIENAAR, 1985; DU PLESSIS & DUNCAN, 1989). Once the plants are a couple of inches high, they are transplanted into individual pots and grown until they are large enough to plant in the garden.

Eucomis species are prone to attack by the Cherry Spot Caterpillar (*Diaphone*). However, there are no other serious pests or diseases that apparently trouble the genus.

1.4.2 Value of *in vitro* culture methods

Although traditional plant-breeding methods for geophytes have been adequate for centuries, tissue culture is a valuable alternative and has several advantages. Conventional propagation methods include natural division, scaling, chipping, scooping, scoring and leaf and stem cuttings. There are two main disadvantages with

these methods. The first is that it is difficult to produce a large number of plants in a short period of time and the second is that diseases can be easily spread from infected plants (KIM & DE HERTOGH, 1997). Tissue culture techniques, however, can overcome such problems.

Some of the advantages for tissue culturing geophytes is that new cultivars, species and recalcitrant species can be rapidly multiplied, virus-free plants can be produced and the technique allows for crop improvement (HUSSEY, 1982a; KIM & DE HERTOGH, 1997; CHANG *et al.,* 2000).

Tissue culture can also aid with the conservation of valuable biodiversity and the micropropagation of whole plants can play a part in the establishment of breeding material from wild populations and in mass-producing material for selection or genetic engineering. Tissue culture often leads to genetic disturbances, which results in somaclonal variation, which can, extend the range of variation for the plant breeder to possibly use the variability for plant improvement (KRIKORIAN & KANN, 1986; DEBERGH, 1994; CHANDLER & LU, 2005; CANTER *et al*., 2005).

Micropropagation also allows for storage of plantlets in a small space, year round multiplication, the avoidance of long dormant seasons and easy international exchange of plant material (HUSSEY, 1980; KIM & DE HERTOGH, 1997). Lastly, controlled culture conditions, both chemical and environmental, makes tissue culture very advantageous. By controlling the conditions, optimal conditions for the production of secondary metabolites, plantlet regeneration and growth can be established.

Therefore, tissue culture techniques can provide plant breeders with a large volume of high quality material that will limit the need for large collection from the field.

1.4.3 Tissue culture of the Hyacinthaceae

The Hyacinthaceae is one of the most horticulturally important families of monocotyledons as the family includes a number of plants that are medicinally and ornamentally important. It is for these reasons that tissue culture has been carried out on various members of the Hyacinthaceae (AFOLAYAN & ADEBOLA, 2004). Members that have been successfully propagated *in vitro* include *Bowiea volubilis, Ornithogalum* spp*., Drimia robusta, Lachenalia* spp. and *Urginea* spp. (HUSSEY, 1976; JHA *et* al., 1984; EL GRARI & BACKHAUS, 1987; COOK *et al*., 1988; JHA *et* al., 1991; LANDBY & NIEDERWIESER, 1992; HANNWEG *et al*., 1996; NGUGI *et al*., 1998; SLABBERT & NIEDERWIESER, 1999; MALABADI & VAN STADEN, 2004).

The choice of explant is an important factor when it comes to the desired outcome. This choice will vary with each family. The source of explants mainly used for the establishment of Hyacinthaceae species *in vitro* is the bulb. A disadvantage with using the bulb as an explant is that it destroys the parent plant. This is a problem in terms of conserving vulnerable species. Another disadvantage is contamination. Contamination of plantlets produced *in vitro* is often associated with the use of the bulb as an explant (ZIV & LILIEN-KIPNIS, 1997; ZIV & LILIEN-KIPNIS, 2000; FENNELL & VAN STADEN, 2004).

Members of Hyacinthaceae that have previously been micropropagated successfully from bulb scales or bulb sections include: *Bowiea volubilis, Charybdis numidica*, *Drimia robusta, Galtonia* spp*, Hyacinthus orientalis, Merwilla* spp., *Muscari* spp., *Ornithogalum* spp., *Schizobasis intricate* and *Urginea* spp. (FENNELL & VAN STADEN, 2004; ASCOUGH *et al.,* 2008). A protocol using twin-scales was developed by AULT (1995) for the tissue culture of *Eucomis autumnalis, E. comosa,* and *E. zambesiaca*.

Inflorescence stalks are a useful alternative for overcoming contamination that is often a major problem when working with underground organs as the explant (ZIV &

LILIEN-KIPNIS, 1997). According to ZIV and LILIEN-KIPNIS (1997), if the inflorescence is isolated at an early stage, before the differentiation of reproductive organs, both the peduncle and pedicel tissue can be induced to form buds, plantlets or bulbs *in vitro*. Other advantages of using floral tissues as explants is that the explant material is obtained at flowering time which allows for the selection of desirable floral traits (O'ROURKE *et al*., 1991; KIM & DE HERTOGH, 1997). Inflorescences produce a large number of potential meristems for propagation and the use of floral explants is important for *in vitro* breeding purposes such as *in vitro* hybridization, *in vitro* pollination, fertilization and embryo rescue (O'ROURKE *et* al., 1991; DEBERGH, 1994). Haploid plants cultured from anthers, pollen grains, ovaries and unfertilized ovules can be used to produce homozygous diploids by doubling the chromosomes such as in *Gladiolus* and *Freesia* (KIM & DE HERTOGH, 1997). One disadvantage of using the inflorescence as explants is that not all bulbs flower every year and so there is a possibility of off-types being produced in some years (DE BRUYN *et al*., 1992). Some members of the Hyacinthaceae family have been micropropagated successfully from floral explants. HANNWEG *et al.* (1996) developed a protocol for the regeneration of *Bowiea volubilis* bulblets from the inflorescence stem and *Ornithogalum dubium* was propagated by using the peduncle (ZIV & LILIEN-KIPNIS, 2000). Flower buds were used as explants to initiate bulbs of *Hyacinthus orientalis* (KIM *et al*., 1981).

Leaf material is an even more suitable source of explant material than bulbs as harvesting is non-destructive to the parent plant (DREWES & VAN STADEN, 1994; ZIV & LILIEN-KIPNIS, 2000; TAYLOR & VAN STADEN, 2001c). Limiting factors when using leaf material as the explant is seasonal availability, young leaves are only available in spring and leaf explants of many species do not respond in culture (TAYLOR & VAN STADEN, 2001c; ASCOUGH *et al.,* 2008).

McCARTAN and VAN STADEN (1998) and McCARTAN and VAN STADEN (2002) successfully established shoots of *Merwilla plumbea* (Syn. *Merwilla natalensis*, *Scilla natalensis*, *Scilla kraussii*) and *Merwilla dracomontana* (Syn. *Scilla dracomontana*) *in*

vitro using leaf explants. *Eucomis pole-evansii* was micropropagated using leaf explants from seedlings germinated *in vitro* (McCARTAN & VAN STADEN, 1995) while TAYLOR and VAN STADEN (2001c) used leaf explants to successfully initiate multiple shoot production in the *Eucomis* species. *Eucomis vandermerwei* was micropropagated by using primary leaf explants which initiated shoots. These shoots then provided a decontaminated source of secondary leaf explants (McCARTAN *et al*., 1999).

Shoots can be used as another source of explant material, however, not many members of the Hyacinthaceae have been propagated using shoot material. Successfully cultured members are *Lachenalia* spp. and *Merwilla plumbea* (ASCOUGH *et al*., 2008).

Micropropagation is a biotechnological tool that is widely used for the bulk propagation of plants. This is useful for the conservation of threatened species and with stimulating the horticultural trade. Protocols have been established for several genera of the Hyacinthaceae (Table 1.1) but techniques and protocols for many members of the family still need to be developed.

Table 1.1: Members of the Hyacinthaceae family that have been propagated through tissue culture (McCARTAN & VAN STADEN, 1999; FENNELL & VAN STADEN, 2004; ASCOUGH *et al.,* 2008).

Plant name	Growth response
Bowiea volubilis	Callus, shoots, bulblets and plantlets
Charybdis numidica	Bulblets
Drimia robusta	Bulblets
Eucomis autumnalis subsp *autumnalis*	Shoots and plantlets
E. autumnalis subsp *amaryllidifolia*	Shoots and plantlets
E. autumnalis subsp *clavata*	Shoots and plantlets
E. bicolor	Shoots and plantlets
E. comosa var *comosa*	Shoots and plantlets
E. humilis	Shoots and plantlets
E. comosa var *striata*	Shoots and plantlets
E. pole-evansii	Shoots and plantlets

E. vandermerwei	Shoots and plantlets
E. zambesiaca	Shoots and plantlets
Galtonia candicans	Shoots
G. viridiflora	Plantlets
Hyacinthus orientalis	Callus, shoots, bulblets and plantlets
Lachenalia	Buds, shoots and plantlets
Merwilla dracomontana	Shoots
M. plumbea	Shoots, bulblets and plantlets
Muscari	Bulblets
M. armeniacum	Bulblets
M. botryoides	Bulblets
M. racemosum	Bulblets
Ornithogalum dubium	Bulblets
O. longibracteatum	Bulblets
O. thyrsoides	Bulblets and plantlets
O. umbellatum	Callus, shoots and plantlets
Schizobasis intricate	Callus, shoots and plantlets
Thuranthos basuticum	Plantlets
Urginea indica	Callus, somatic embryos, shoots, bulblets and plantlets
U. maritime	Bulblets and plantlets
Veltheimia bracteata	Shoots and plantlets

1.4.4 A review of the micropropagation schemes for bulbous plants

Explants

For *in vitro* regeneration and growth of bulbous plants, scales from the basal regions where they are joined to the basal plate, stems and buds that have already differentiated, have been found to be the most successful explants. However, the choice may depend on the family to which the bulb belongs (FENNELL & VAN STADEN, 2004,). With regards to the Hyacinthaceae family, the most successful cultures have been established *in vitro* from bulb sections or bulb scales.

Existing shoot meristems in the form of axillary buds may be dissected out of bulbs and cultured directly. Other organs such as leaves, stems and parts of the inflorescence can also be used and are reported to be useful for the induction of adventitious shoot meristems. Callus formation can be used to obtain shoots

(HUSSEY, 1980; HUSSEY, 1982a). ZIV and LILIEN-KIPNIS (1997) reported that the inflorescence stalk is a very good source for regenerative explants and ZIV and LILIEN-KIPNIS (2000) showed that buds can be regenerated *in vitro* from various types of inflorescence such as the umbel or spike.

Explant orientation has also been said to play a role in bulblet regeneration. LESHEM *et al.* (1982) observed that explant orientation in *Lilium longiflorum* greatly affected regeneration percentage. Scale sections that were placed adaxial side down regenerated fewer and smaller bulbs, less roots but produced more callus than scales that were abaxial side down on the medium. JACOBS *et al.* (1992) also examined the effect of orientation on *Nerine bowdenii*. Explants that were orientated abaxial side down formed bulblets more readily than adaxial orientated explants. This was also found to be true by TAYLOR and VAN STADEN (2001c) with *Eucomis* species. Fewer shoots initiated on explants when the adaxial side of the leaf explant was placed in contact with the culture medium.

The effect of growth regulators

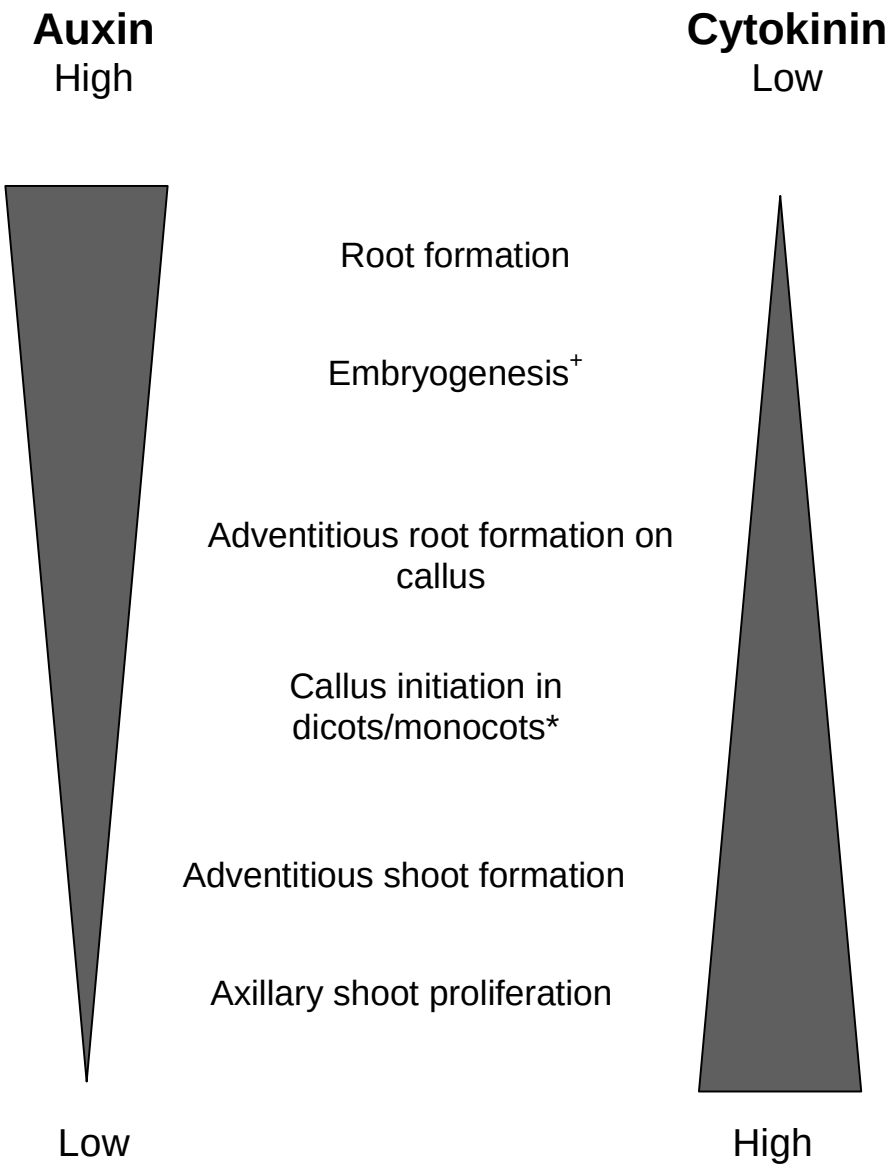
Responses of explants vary considerably. The vigour and type of reaction depends on the presence and concentrations of plant growth regulators. Auxins and cytokinins are the two most important and widely used hormonal supplements. Cytokinins and auxins interact to affect differentiation. A high auxin to low cytokinin ratio stimulates root development while a low auxin and high cytokinin ratio promotes bud and shoot development. Equal concentrations of auxins to cytokinins result in callus formation (Figure 1.1).

Auxins influence plant growth in many ways including cell enlargement and elongation, apical dominance, abscission of plant parts, tuber and bulb formation and adventitious rooting. The commonly used auxins are napthaleneacetic acid (NAA), indole-3-acetic acid (IAA) and indole-butyric-acid (IBA). Cytokinins are mainly

associated with *in vitro* shoot proliferation but they also promote cell division and influence cell enlargement and tissue differentiation (DAVIES, 1987).

Low levels of auxin produce swelling of the explant and very few shoots are produced. However, FENNELL and VAN STADEN (2004) claimed that with regards to bulbous plants, low concentrations of auxin increase the induction of cell division, leading to shoot production. High auxin concentrations are often used to induce callus and shoots may form on explants weeks later (HUSSEY, 1977). The type of auxin used is important with regards to the production of callus type.

Media containing both cytokinin and auxin result in the formation of multiple shoots on the explant and most monocotyledons require both these growth regulators for maximum shoot production. Cytokinins enhance axillary and adventitious shoot formation. When bulbs are used as the explant source, cytokinins may not be essential (FENNELL & VAN STADEN, 2004) and the study carried out by PIERIK and STEEGMANS (1975) on *Hyacinth* showed that cytokinins had hardly any effect on bulblet regeneration and bulblet weight. HEMPEL (1979) and HUSSEY (1982b) reported that *Lilium*, *Narcissus* and *Hyacinthus* plantlets can be produced in the absence of plant hormones.



+ Organogenesis in monocotyledons is often promoted in a medium without auxin or reducing the auxin concentration.

* In monocotyledons callus induction may require high levels of auxin, cytokinin may be unnecessary.

Figure 1.1: Graphical representation of the various effects of cytokinins and auxins on *in vitro* cultures (GEORGE & SHERRINGTON, 1984).

Media and Culture Conditions

Nutrient media for plant tissue culture were developed to allow explants to grow in an artificial environment. Media were developed that contained the essential elements for plant growth and development that would have usually been available in the soil. The essential elements are divided into two groups: the macroelements and the microelements. Along with the macro- and microelements, the nutrient media contains vitamins, plant growth regulators and a carbon source.

The macroelements, are elements required by the plant in large quantities. These include calcium, magnesium, nitrogen, phosphorous, potassium and sulfur (Table 1.2) The microelements used in the tissue culture media are boron, cobalt, copper, iodine, iron, manganese, molybdenum and zinc (Table 1.2). Microelements are needed in small quantities.

Table 1.2: The constituents of a modified Murashige and Skoog (1962) basal medium.

Components	Salt(s)	Mass / litre stock (g)	Volume stock (ml)/L final medium	Stock solution no.
Macro-Elements	NH_4NO_3	165	10	1
	KNO_3	190	20	2
	$CaCl_2.2H_2O$	44	10	3
	$MgSO_4.7H_2O$	37	10	4
	NaFeEDTA	3.73	10	5
	KH_2PO_4	17	10	6
Micro-Elements	H_3BO_3	0.62	10	7
	$MnSO_4.4H_2O$	2.23		
	$ZnSO_4.7H_2O$	0.86		
	KI	0.084		

	$Na_2MoO_4.2H_2O$	0.025		
	$CuSO_4.5H_2O$	0.0025	10	8
	$CoCl_2.6H_2O$	0.0025		
Vitamins	Thiamine HCl	0.01	10	9
	Nicotinic acid	0.05		
	Pyridoxin HCl	0.05		
	Glycine	0.20		

In addition: Myo-inositol 100 mg/L
Sucrose 30 g/L (3%)
Agar 8 g/L (0.8%)

TAKAYAMA and MISAWA (1979) studied the effect the strength of MS medium had on bulblet formation on *Lilium auratum* and *Lilium speciosum*. Formation and growth of bulbs and roots were affected by the strength of MS medium. The number of bulbs and bulb weight increased with an increase in MS medium strength. Root number and weight was not influenced by the MS medium strength. However, root length was inhibited by an increased MS strength. BONNIER and VAN TUYL (1997) reported that lily bulblet and sprout growth at 25 ℃ was significantly reduced by using ¼ strength MS medium compared to full strength MS medium, while VAN DER LINDE *et al.* (1988) reported that the optimum growth condition for *Iris hollandica* is 20 ℃ in the dark on ½ strength MS.

Carbohydrates serve as the energy source for plant tissue cultures and they are essential for plantlet growth and adventitious root formation. Although plant tissues have internal reserves of carbohydrates, an external supply is provided by either adding sucrose to the media or through photosynthesis in green tissues (ECONOMOU & READ, 1987). Sucrose is considered to be the best source of carbon for *in vitro* tissue culture with optimum concentrations generally being

between 30 and 60 g/L. This, however, may vary among plant species with some plants needing higher or lower concentrations.

Plant morphogenesis is affected by environmental factors such as temperature, carbon dioxide, nutrients and light (DA SILVA & DEBERGH, 1997). Light can have an effect on the morphology of the cultured plants, however a plant's response to light will vary depending on the light intensity, duration and quality. Light quality has an important effect on morphological characteristics such as plant elongation, axillary shoots and leaf anatomy. Light intensity regulates leaf and stem size and morphogenic pathways. There are great differences with regards to the effect of illumination on *in vitro* bulblet formation for different bulbous plants. STEINITZ and YAHEL (1982) found that continuous darkness increased bulblet size and number during the micropropagation of *Narcissus*. Contradictory to this, continuous light promoted bulblet production of *Lilium japonicum* (MAESATO *et al*., 1994), whereas LESHEM *et al.* (1982) and SLABBERT and NIEDERWIESER (1999) found no difference in the effect of light or dark on bulblet formation in *Lilium longiflorum* and *Lachenalia* respectively. Therefore, this emphasizes the effect of explant material (variety, age, time of culture and so on) on regeneration and growth *in vitro* of bulbous plants (SLABBERT & NIEDERWIESER, 1999).

Temperature plays a pivotal role in the tissue culture environment influencing many responses of the cultured plants. Cultures are generally maintained in an environment where the temperature is 25 ℃ ± 2 ℃, however, optimum temperatures for growth vary with the species and the stage of development.

Methods of multiplication

Multiplication *in vitro* can be the result of either organogenesis or embryogenesis and both these processes may occur directly from the explant or indirectly from callus tissue. Multiplication of higher plants through callus is very difficult and the cultures are genetically unstable (PIERIK, 1991). Therefore, the most common multiplication

method and the most successful method with regards to the Hyacinthaceae and the multiplication of bulbs, is direct organogenesis.

In vitro regeneration and growth of bulbs have been reported using bulb scales (FENNELL & VAN STADEN, 2004). Bulblet growth was stimulated by transferring the bulb scales into liquid medium and shake-culture (TAKAYAMA *et al*., 1991). Liquid culture systems are becoming more popular with regards to mass propagation as well as offering many other benefits. Liquid culture is more cost effective as a gelling agent is not required and it reduces the labour intensive manipulation for plantlet transfer and sub-culturing. Agitation enriches the culture medium with oxygen by facilitating better mixing which allows for better adsorption of the medium constituents and so liquid cultures exhibit higher multiplication and proliferation rates (VARSHNEY *et al.,* 2000; ASCOUGH & FENNELL, 2004). The growth and multiplication of bulbous plants in liquid cultures generally occurs more rapidly, with an increase in the number of buds produced and better bulblet growth, however, this is not always the case (ZIV, 1997; FENNELL & VAN STADEN, 2004).

According to MORAN *et al.* (2003), the use of liquid medium supplemented with sucrose accelerates biomass gain and the bulbing of small shoots of *Cyrtanthus clavatus* and *Cyrtanthus spiralis*. Therefore, the use of liquid culture in order to obtain a high number of bulbs is a good alternative to the conventional methods.

Bulb induction and growth

Once vigorously growing shoots are obtained it is reported that bulblets form spontaneously. Bulbs can however, be induced by varying a number of factors. This includes the transfer of shoots to a medium containing a high level of sucrose, high temperatures between 15 and 30 ℃, darkness, low pH , low concentrations of auxins and charcoal (ZIV, 1991).

NIIMI *et al.* (1999) studied the effects of temperature and light conditions on excised scales of lily bulbs from several *Lilium* species. The study showed that temperature and light do affect *in vitro* regeneration and that more bulblets were regenerated at a higher temperature of 25 ℃. With regards to the effect of light, the different species produced different results. In *Lilium longiflorum*, the number and size of bulbs increased in the dark and suppressed under a 16 h photoperiod, whereas *Lilium japonicum* and *Lilium speciosum* responded best under a 16 h photoperiod.

Certain plants such as lilies may develop dormancy depending on the culture conditions. LANGENS-GERRITS *et al.* (2003) reported that dormancy can be broken by storage for a few weeks at a low temperature. The effect of low temperature on sprouting, time of leaf emergence and further bulb growth was studied. Not only did the low temperature affect the number of sprouted bulblets but also the time of emergence. Generally bulblets grew faster after a low temperature treatment for six weeks (LANGENS-GERRITS *et al.,* 2003).

1.4.5 Future prospects

The increase in the trade of both ornamental and medicinal plants in South Africa is having a negative impact on the wild populations and is actually threatening the survival of many species. Conventional propagation methods have proven to be inadequate with regards to mass propagation of over-exploited plants. Therefore, *in vitro* plant regeneration could be a potential solution to this problem. Successful micropropagation protocols for certain species of the Hyacinthaceae family (Table 1.1) have already been developed and hopefully in the near future, protocols for many other species will be developed.

1.5 AIMS AND OBJECTIVES

The aim of this study was to establish a protocol for the optimization of *in vitro* bulblet induction in *Eucomis zambesiaca*. Thus the specific objectives of this study were to investigate the effect of environmental and physiological parameters on the initiation and growth of bulblets. These included the effect of temperature, photoperiod, various carbohydrates at different concentrations and combinations as well as various plant growth regulators.

Chapter 2
TEMPERATURE AND ORIENTATION

2.1 INTRODUCTION

Temperature and light play a pivotal role in the tissue culture environment influencing many responses of the cultured plants. Temperature is a natural regulator of plant growth and morphogenesis. It not only regulates growth rates but also the transition between various vegetative and reproductive phases during development (ASCOUGH *et al*., 2008). Cultures are generally maintained in an environment where the temperature is kept constant. This temperature is usually 25 ℃ ± 2 ℃. The problem with this is that it does not recognize the diurnal and seasonal temperature fluctuations under which a plant would normally develop (MURASHIGE, 1974). Although constant temperatures may be adequate for the tissue culture of certain plants (mainly annuals and tropical species which grow in relatively uniform temperature conditions) more research is needed to investigate the influence on temperature of plants that are adapted to more temperate and desert climates. MURASHIGE (1974) suggests that maximum success from tissue culture may only be achieved when the precise temperature needs of a plant are fulfilled.

Temperature can be useful with regards to long term *in vitro* storage. Long term storage is important for the improvement of crops. Therefore, plant breeding companies and research institutes are examining methods for the long term storage of plants. For long term *in vitro* storage, plant material can be kept under conditions that result in slow growth or it can be cryopreserved. It is believed that slow growth *in vitro* may be obtained by low temperature (BONNIER & VAN TUYL, 1997). Both KELLER (1992) and BONNIER and VAN TUYL (1997) studied the effects of temperature on the *in vitro* storage of onion and lily. KELLER (1992) found that -2 ℃

was as favourable as +2 ℃ for storage. Treatments at 45 ℃ damaged plants and inhibited growth in onion and rakkyo lines.

Low temperatures (0 to 10 ℃) are not used to store lilies as dormancy is broken and the bulblets sprout during long storage even under these low temperatures. Therefore, lily bulbs are generally stored at a temperature of -2 ℃. A temperature of 25 ℃ has however been reported to induce dormancy in different lily species (BONNIER & VAN TUYL, 1997). Bulblet viability and regrowth percentage was highest when bullets were stored at 25 ℃. At -2 ℃ bulblet growth was very low. Therefore, BONNIER and VAN TUYL (1997) believe that storage of lily bulblets at 25 ℃ is a promising method to preserve lilies.

Temperature is considered to be a major factor in the regulation of dormancy. It has been reported that high gibberellin concentration, low sucrose concentration and low temperature during *in vitro* culture reduces the level of dormancy. DELVALLÉE *et al.* (1990), therefore, studied the development of dormancy in bulblets of *Lilium speciosum*. At 15 ℃, bulblets were viable and little or no dormancy was induced compared to 20 ℃ where bulblets were viable but dormant. The onset of dormancy was earlier and the rate of development of dormancy was higher at 25 ℃ than at 20 ℃. LANGENS-GERRITS *et al.* (2003) reported data that is in agreement with DELVALLÉE *et al.* (1990). Bulblets were also not dormant at 15 ℃ while at 20 and 25 ℃ bulblets were dormant. Bulblets at 20 and 25 ℃ needed a cold treatment of 5 ℃ for at least 6 weeks to break dormancy. The average bulb weight at 15 ℃ was 45 mg while at 20 ℃ it was 75 mg. LANGENS-GERRITS *et al.* (2003) reported that dormant and non-dormant bulblets grew the best after a low temperature treatment while there was very little growth in bulblets that had not been pre-exposed to a low temperature.

VAN DER LINDE *et al.* (1988), TAEB and ALDERSON (1990) and GRASSOTTI (1997) all looked at the effect of a low temperature treatment on bulblet development. VAN DER LINDE *et al.* (1988) studied *Iris hollandica* and discovered that the

optimum growth condition is 20 ℃ in the dark on ½ strength MS media. At 15 and 20 ℃ there was 90-100% regeneration of bulblets while at 25 ℃ there was no regeneration. An interruption of the 20 ℃ culture condition by a period of 4 weeks at 5 ℃ enhanced bulb formation and bulb weight. TAEB and ALDERSON (1990) found that low temperature incubation of tulip shoot cultures enhanced bulb development. Shoots that underwent a cold treatment produced a high number and high percentage of bulbing shoots compared to those with no cold treatment. TAEB and ALDERSON (1990) also discovered that the number of shoots that formed bulbs was significantly affected by an interaction between sucrose levels and temperature. In *Lilium* x 'Polyanna' and *Lilium longiflorum,* when incubated at 22 ℃ and followed by a cold treatment of 0 ℃, number of bulblets decreased but bulblet weight and size increased. Therefore, the results show that temperature during culture incubation is an important factor determining the number, weight and size of bulbs produced during propagation (GRASSOTTI, 1997).

Adventitious buds and roots of *Streptocarpus* x *hybridus* 'Constant Nymph' formed when leaf discs were stimulated by low temperatures such as 12 and 18 ℃ (APPELGREN & HEIDE, 1972). APPELGREN and HEIDE (1972) found that increasing temperature decreased bud and root formation. At 30 ℃ bud and root formation was very poor, while the highest number of organs formed at 12 and 18 ℃. APPELGREN and HEIDE (1972) concluded that striking differences exist in the ability of various plant species to regenerate new organs depending on temperature.

TAKAYAMA and MISAWA (1979) and YAMAGISHI (1998) both studied the effect of temperature on *Lilium* species. YAMAGISHI (1998) looked at *Lilium japonicum* and reported that the enlargement of bulbs and bulb weight was higher at 20 ℃ compared to 15 and 26 ℃, which suppressed rooting. These results confirmed the results of MAESATO *et al.* (1994), who showed that 20 ℃ was more suitable than 25 ℃ to enlarge *in vitro* bulblets of *L. japonicum*. It was concluded that culturing at the most suitable culture temperature is important for bulb enlargement. TAKAYAMA & MISAWA (1979) tested a range of temperatures from 20 to 30 ℃ on *Lilium*

auratum and *Lilium speciosum.* The optimum temperature for differentiation and growth was 20 ℃. Culture temperature has a strong effect on the growth of *in vitro* bulblets of *Lilium* species (TAKAYAMA & MISAWA, 1979; YAMAGISHI, 1998).

The optimum temperatures for bulblet growth for both *Nerine bowdenii* and *Heloniopsis orientalis* were 21 to 25 ℃ (PIERIK & IPPEL, 1977; KATO & OZAWA, 1979). Bulblet formation of *N. bowdenii* was strongly promoted by raising the temperature, with an optimum at 21 to 25 ℃, for both number of bulbs and bulblet weight (PIERIK & IPPEL, 1977). JACOBS *et al.* (1992) found the optimum temperature for bulblet production of *N. bowdenii* was 17 to 22 ℃. The highest fresh weight per bulblet and total fresh weight of bulblets produced per explant was at this temperature. High temperatures were found to reduce the percentage of explants that formed bulblets. These results confirmed the data of PIERIK and IPPEL (1977). Bud formation was best at a temperature of 21 to 25 ℃, however, a pretreatment at 16 ℃ for 7 to 21 days increased bud formation compared to a constant temperature of 16 and 25 ℃. A 30 ℃ pretreatment decreased the number of buds while at both 16 and 30 ℃ only a few shoots developed. A continuous treatment at 30 ℃ inhibited bud formation. Lastly, at 30 ℃ the survival rate was 60% while at 16, 21 and 25 ℃ there was 100% survival rate (KATO & OZAWA, 1979).

BROOKING *et al.* (1997) looked at the effect of temperature on tuber growth in *Sandersonia aurantiaca*. Tuber growth rates were significantly affected by temperature. The maximum tuber fresh weight was obtained at 21 ℃. The incidence of secondary tubers was also influenced by temperature. Very few formed at temperatures of 15 and 18 ℃ but increased at 21 ℃ and higher. The maximum number was formed at 27 ℃. BROOKING *et al.* (1997) discovered that low temperatures maximized tuber weight but minimized secondary tuber incidence.

The induction and formation of tulip bulblets was affected by temperature and light quality. It was found that plantlets which were exposed to white light and cultured at 25 ℃ before a chilling period resulted in a higher crop of bigger bulbs compared to

tulips cultivated in red light. White light at a temperature of 20 °C was less suitable for bulblet formation and growth, which resulted in a lower percentage of large bulblets in comparison to 25 °C (BACH & PAWLOWSKA, 2006).

From the investigations carried out by DE CAPITE (1955) it was concluded that the growth of *in vitro* cultures is strongly influenced by temperature. He found that the best results were obtained when plant material was grown under a constant temperature throughout their life period. This is in contradiction to research by BACH *et al*. (1992a) for *Hyacinthus orientalis*. BACH *et al.* (1992a) found that if *H. orientalis* was continuously cultured at the same temperature, the adventitious shoots did not form bulblets. However, if the shoots were given a low temperature treatment and transferred to 23 °C, then the development of bulblets occurred.

SEABROOK and CUMMING (1982); PIERIK *et al.* (1983); NIIMI *et al.* (1999) and KULKARNI *et al.* (2005) found 25 °C to be the best temperature for shoot and bulblet growth. The largest number of shoots and bulbs of *Narcissus* was formed at 25 °C while the leaf width was greatest at 30 °C but the leaves were abnormal with wrinkled, curled and uneven surfaces. *Narcissus* shoots were significantly greater at a constant temperature of 25 °C compared to other constant temperatures and alternating temperatures tested (SEABROOK and CUMMING, 1982). These findings agree with DE CAPITE (1955). PIERIK *et al.* (1983) looked at the propagation of *Eucharis grandiflora* and discovered that regeneration and bulblet weight were promoted by raising the temperature from 21 to 25 or 29 °C. Rooting and leaf formation were increased by increasing the temperature from 17 to 25 °C.

With regards to *Lilium* spp., more bulblets regenerated as well as grew better at a temperature of 25 °C than at 15 °C. At 15 °C bulblets tended to rot. Bulblets grown at 25 °C sprouted more often once planted in the greenhouse (NIIMI *et al.,* 1999). KULKARNI *et al.* (2005) looked at the temperature requirements for seedling growth of two Hyacinthaceae species. Different temperatures affected the survival and seedling growth of both species; *Albuca pachychlamys* and *Drimia robusta*. In *A.*

pachychlamys highest seedling survival rate was at 10 and 15 ℃. Seedling survival was low at 25 ℃, however, seedlings had more roots and higher total seedling and bulb mass. *D. robusta* seedlings grew best at 25 ℃ and seedling and bulb mass were greatest at this temperature. At 10℃ and 35 ° C, 36% and 32% seedlings survived respectively. Therefore, KULKARNI *et al.* (2005) concluded that *A. pachychlamys* should be grown at low temperatures for a few days and then transferred to higher temperatures, not exceeding 25 ℃, while *D. robusta* can be grown at 25 ℃.

FANIZZA *et al.* (1988) and MARTIN-CLOSAS and PELACHO (1997) looked at the influence of high temperatures on root induction and tuberization. FANIZZA *et al.* (1988) found that at 25 ℃ no roots were induced in *Vitis vinifera*, however, 32 and 35 ℃ were effective in inducing roots in the shoot tip cultures and the shoot tip developed into a single rooted shoot. At 38 ℃ the shoot tips died. High temperatures are inhibitory to tuber induction and development, and so MARTIN-CLOSAS and PELACHO (1997) studied the increase in potato tuberization and growth by jasmonic acid (JA) at high temperatures. Explants grown at 30 ℃ on media containing low concentrations of JA increased tuberization, weight and elongation compared to explants grown at 23 ℃. From their experiments, MA RTIN-CLOSAS and PELACHO (1997) learnt that the effect of limiting temperatures for potato plant development can be modified by JA treatments.

The temperature affects the type of morphogenesis during *in vitro* culture. It has been found that the optimum temperature for the morphogenetic response varies with species and cultivars.

Explant orientation has also been said to play a role in bulblet regeneration. LESHEM *et al.* (1982) studied the effect of explant orientation on the regeneration and subsequent growth of bulblets on *Lilium longiflorum*. It was observed that explant orientation greatly affected regeneration percentage. Scale sections that were placed adaxial side down regenerated fewer and smaller bulbs, less roots but produced

more callus than scales that were abaxial side down on the medium (LESHEM *et al.,* 1982). JACOBS *et al.* (1992) also examined the effect of orientation on *Nerine bowdenii*. Explants that were orientated abaxial side down formed bulblets more readily than adaxial orientated explants. The number of bulblets formed was more for adaxial than abaxial-orientated explants, however, the average weight per bulblet for abaxial-orientated explants was more than double that for adaxial-orientated explants (JACOBS *et al.,* 1992).

TAYLOR and VAN STADEN (2001c) found that explant orientation affected the regenerative potential of the leaf tissue in *Eucomis* species. Fewer shoots initiated on explants when the adaxial surface was placed in contact with the medium. Similar results were obtained for *L. longiflorum* bulblet production from bulb scales (LESHEM *et al.,* 1982).

Hyacinthus orientalis cv. Carneige was studied by YI *et al.* (2002) and it was discovered that the highest percentage of bulblet regeneration occurred when explants were placed abaxial side down while the lowest occurred on adaxial orientated explants. From these experiments YI *et al.* (2002) reported that explant orientation may be less involved in root formation and that growth regulators actually play a greater part in root development. It has been found that in hyacinth the normal orientation (abaxial) may be more effective for bulblet formation and root development than the inverted orientation (adaxial). It has however been reported that when explants were placed adaxial side down on the media, bulblet formation and growth was enhanced (PIERIK & POST, 1975). These findings are not in agreement with the results obtained by YI *et al.* (2002). However, PAEK and THORPE (1983) confirmed the results of YI *et al.* (2002), as they also found that the number of bulblets formed on the abaxial side of explants was higher than the adaxial orientated explants of hyacinth.

2.2 MATERIALS AND METHODS

Cultures of *Eucomis zambesiaca* Baker. plants were established using the protocol of TAYLOR and VAN STADEN (2001c). The cultures were bulked up and subcultured on MURASHIGE and SKOOG (1962) medium supplemented with 100 mg/L myo-inositol, 30 g/L sucrose and solidified with 8 g/L agar. The established plants were used to determine the effect of explant orientation and temperature on bulb induction. Leaf material was placed such that either their abaxial or adaxial surfaces were in contact with the solid MS (1962) medium. The above MS medium was used and no hormones were added. The pH of the medium was adjusted to 5.8 with diluted NaOH before autoclaving at 121℃ and 103 kPa for 20 min. Five pieces of leaf material (1 cm) were placed on 40 ml medium in each culture bottle (6 cm diameter, 10 cm high) and there were eight bottles per temperature. The bottles were incubated in growth chambers (Convirons) at temperatures of 10, 15, 20, 25 and 30 ℃ with a 16 h photoperiod. The Convirons contained Osram L58W/640 cool white fluorescent bulbs. The light intensity was measured using a radiation meter and the Conviron had a light intensity range of 70 to 90 µmol/m^2/s.

As five pieces of leaf material were placed in each bottle and there were eight bottles per temperature, there was a total of forty replicates. Bulblets were collected, counted and weighed together, therefore, the results for weight is bulblet weight per jar, not individual bulbs. Data collected were subjected to one-way analysis of variance (ANOVA). Where there is significant difference (p=0.05), the means were separated using Duncan Multiple Range Test (DMRT). Data analysis was done using SPSS version 15.0.

2.3 RESULTS

After a month in culture, small shoots were visible. These shoots swelled at the base and formed bulblets after a further two months (Figure 2.1). The healthiest bulblets were produced at 20 and 25 ℃.

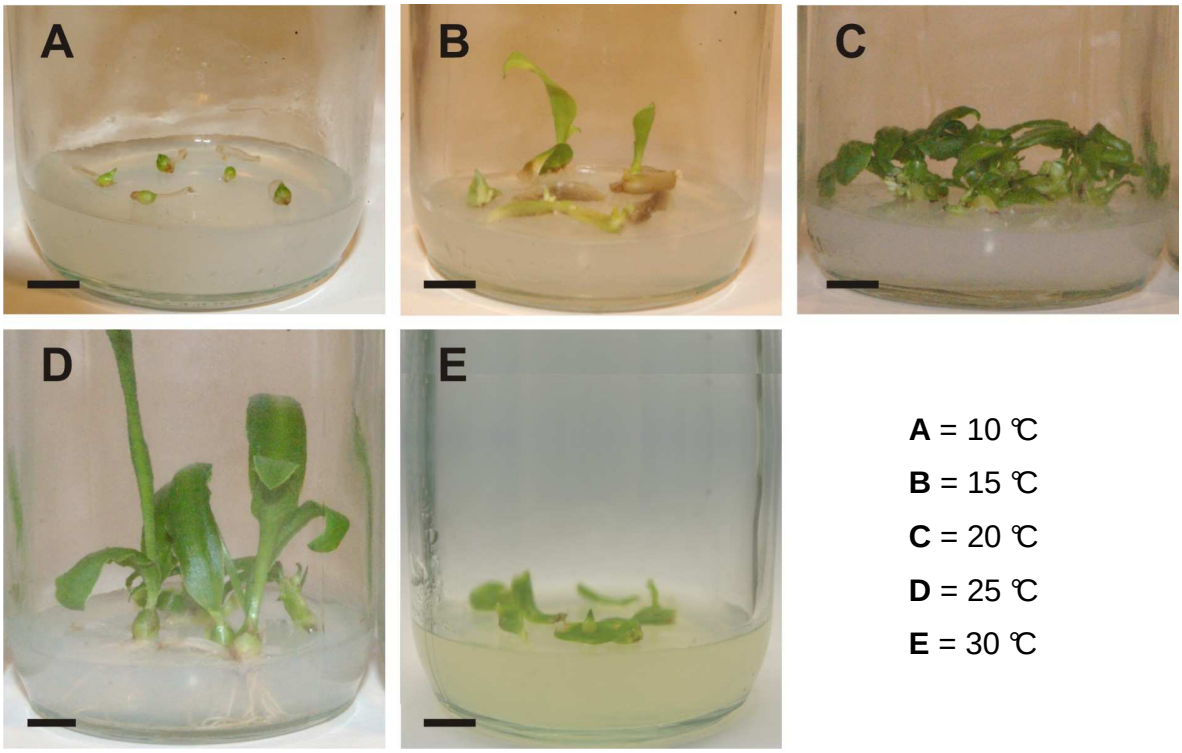


Figure 2.1: Effect of different temperatures on bulblet induction from abaxially-orientated leaf explants of *E. zambesiaca*. Bar = 1 cm.

The highest mean bulblet weight per jar was obtained at 20 ℃, while the next highest value was from 25 ℃ (Table 2.1).

Table 2.1: Effect of different temperatures and explant orientation on the mean bulblet weight per jar of *E. zambesiaca.*

Temperature (℃)	Abaxial (mg)	Adaxial (mg)
10	49.50 ± 15.00^{b}	31.75 ± 14.00^{c}
15	135.50 ± 83.00^{b}	29.75 ± 25.00^{c}
20	782.00 ± 163.00^{a}	658.00 ± 105.00^{a}
25	232.75 ± 59.00^{b}	297.50 ± 30.00^{b}
30	82.50 ± 73.00^{b}	30.25 ± 5.00^{c}

Mean values in the same column followed by the same letter are not significantly different (p=0.05).

For all explants placed adaxial side down, bulblets grew downwards into the medium. At 20 ℃, significantly more bulblets developed fro m abaxially-orientated explants than from adaxially-placed leaf segments (Figure 2.2).

Explants cultured at 20 ℃ produced more bulblets w ith smaller leaves, whereas at 25 ℃ fewer, smaller bulblets with larger leaves we re formed (Figure 2.1 and Figure 2.2). Very few small bulblets were produced on leaf explants grown at 10, 15 and 30 ℃, with an average of less than 1 bulblet formi ng per explant (Figure 2.2).

In order to determine the optimum treatment, the percent bulb induction was converted to a proportion and multiplied by the mean bulb mass to enable a more consistent ranking of treatments (ASCOUGH, 2008). These values are presented in Table 2.2, where it can be seen that 20 ℃ had the highest product value of 57.48. This indicates that the optimum temperature for bulblet induction in *E. zambesiaca* is 20 ℃.

Table 2.2: Effect of temperature on bulblet formation in *E. zambesiaca* and the product value.

Temperature (℃)	Product value
10	7.20
15	7.15
20	57.48
25	32.40
30	8.51

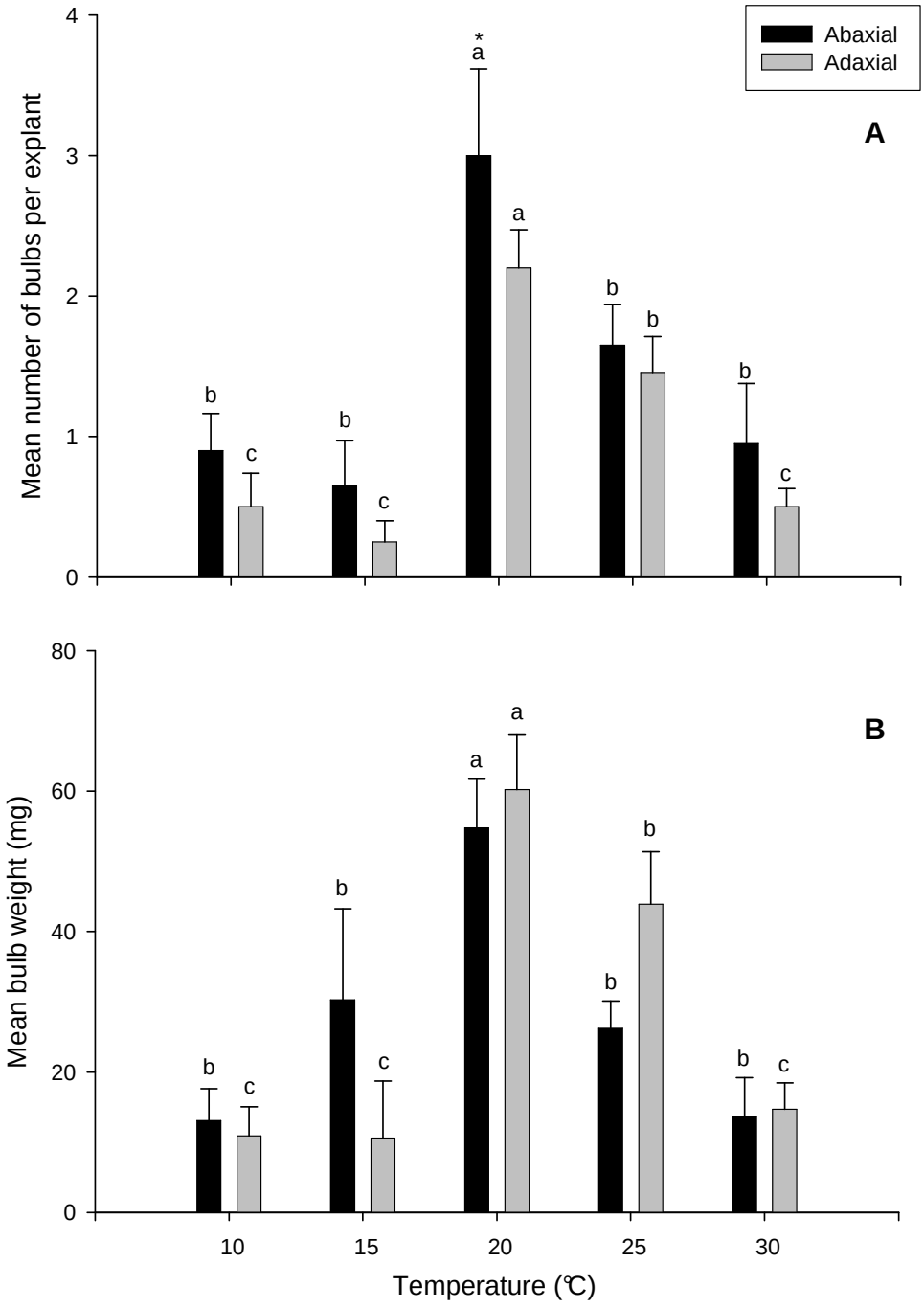


Figure 2.2: Mean bulblet number per *E. zambesiaca* leaf explant (A) and mean bulblet weight (B) affected by various temperatures and explant orientation. Mean values of the same orientation (abaxial/adaxial) with the same letter are not significantly different (p=0.05). Asterisk indicates significant difference between abaxial/adaxial orientation (p=0.05).

2.4 DISCUSSION

From Figure 2.2 it can be seen that 20 ℃ produced the best results for bulblet induction. Looking at the other temperatures it can be observed that there is a definite bell-shaped curve. The lowest values were obtained from the extreme temperatures namely 10 and 30 ℃. At 20 ℃ the high est values were obtained for bulblet number and bulblet weight. This shows that *Eucomis zambesiaca* does not grow well under either a low or a high temperature but rather at a moderate temperature (Figure 2.2).

At 20 ℃ the bulbets that formed had smaller leaves , however, the number of bulblets per explant was higher than at any other temperature tested. Figure 2.2 shows the mean number of bulblets formed per explant and it is clear that with a value of 3.00, 20 ℃ with abaxial placement was the best treatment . This data is in agreement with TAKAYAMA and MISAWA (1979) and YAMAGISHI (1998) who both studied the effect of temperature on *Lilium* species and found 20 ℃ to be the optimum temperature.

Bulblets incubated at 25 ℃ were fewer in number. T hey were, however, larger plantlets than those produced at 20 ℃ (Figure 2.1) . It could be beneficial to initiate bulblet induction and growth at 20 ℃ and then tran sfer them to 25 ℃, so that plantlet size can increase. KULKARNI *et al.* (2005) found that with *Albuca pachychlamys* (Hyacinthaceae) seedling survival was higher 10 and 15 ℃, however, seedling and bulblet mass was greatest at 25 ℃. So it was sugge sted that *A. pachychlamys* should be grown at low temperatures for a few days and then transferred to higher temperatures. This could possibly be beneficial for *E. zambesiaca* which also belongs to the Hyacinthaceae family.

It is also only at 20 ℃ that a significant differe nce was seen between abaxial and adaxial orientation. Abaxial orientation on the medium produced 3.00 bulbs per explant compared to 2.20 bulblets from adaxial orientation (Figure 2.2). This is in

agreement with the work of LESHEM *et al.* (1982), JACOBS *et al.* (1992) and TAYLOR and VAN STADEN (2001c) who all found that abaxial orientation produced more bulblets or shoots. When looking at the other treatments abaxial orientation also produced more bulblets but there was no significant difference found between the different explant orientations. Explant orientation was tested in all experiments carried out for temperature and other effects (light, carbohydrates and hormones). As no significant differences were observed, the results in the following chapters represent combined data for abaxially- and adaxially-orientated explants.

With regards to bulblet weight per jar, a temperature of 20 ℃ yielded bulblet weights (782 and 658 mg) that were double, if not triple, those of the other temperatures (Table 2.1). This is in agreement with MAESATO *et al.* (1994), who showed that 20 ℃ was more suitable than 25 ℃ to enlarge *in vitro* bulblets of *Lilium japonicum,* along with TAKAYAMA and MISAWA (1979) and YAMAGISHI (1998) who also found that 20 ℃ produced bulblets with the heaviest weig ht.

The product value was calculated (Table 2.2) to confirm the results from Table 2.1 and Figure 2.2 which showed that 20 ℃ was the best temperature for bulblet induction in *E. zambesiaca*. A product value of 57.48 was obtained at 20 ℃ wh ile 25 ℃ had the next highest value of 32.40. This cle arly indicates that 20 ℃ is the optimum temperature treatment for bulblet induction. Therefore, in all subsequent experiments, culture bottles were placed in Convirons at 20 ℃.

2.5 SUMMARY

- Abaxially-orientated explants incubated at 20 ℃ for three months produced the highest number of bulblets per explant
- 20 ℃ abaxially-orientated explants had the highest bulblet weight (782 mg) per jar
- 20 ℃ had the highest product value (57.48) and is therefore, the optimum treatment

Chapter 3
PHOTOPERIOD

3.1 Introduction

Several environmental factors can affect bulbous plant growth and development, but the major ones are light and temperature. Plant growth and development depend on light for photosynthesis and photomorphogenesis. Plant tissue cultures also require light, to a lesser degree, to regulate morphogenetic processes. The effect of light on photosynthesis of *in vitro* cultures is of less importance than that of *in vivo* plants. In tissue culture, photosynthesis is not actually necessary since a carbohydrate source is provided (MURASHIGE, 1974). According to ECONOMOU and READ (1987) light influences the success of micropropagation through its three parameters of duration (photoperiod), illuminance (light intensity) and spectral quality (wavelength).

These three light factors affect shoot growth and morphogenesis in addition to having a role in photosynthesis. Light intensity regulates the size of leaves and stems, as well as their morphogenic pathway and is involved in pigment formation and hyperhydricity. Light quality has been reported in different plant cultures to play an important role on several morphologic characteristics. These include plant elongation (e.g. chrysanthemum and tomato), axillary shoots (e.g. grapevine), leaf anatomy and leaf size (e.g. birch) as well as rhizogenesis (e.g. pear) (MURASHIGE, 1974; DA SILVA & DEBERGH, 1997).

Experiments were conducted by ECONOMOU and READ (1987) which looked at light treatments to improve efficiency of *in vitro* propagation systems. All three parameters: duration, illumination and quality were looked at and it was reported that both root and shoot formation was affected by light duration (ECONOMOU & READ, 1987). The photoperiod extends from 0 hours of light (continuous darkness) to 24 hours of light (continuous light). Plant species and explants respond differently to

photoperiods, however, most plants need a photoperiod of 8 to 18 hours of light from cool white light bulbs (ECONOMOU & READ, 1987).

Table 3.1: Results from light duration experiments carried out to test the effect on shoot and root formation (ECONOMOU and READ, 1987).

Shoot Formation	
Photoperiod	**Effect on culture**
Continuous darkness	• Promoted shoot regeneration in leaf explants of *Crassula argentea* • Increased bulb number and size in scale segments of *Lilium longiflorum* • Promoted bulblet regeneration and production in *Hyacinthus orientalis* bulb scale segments
16 h / 8 h light/dark	• Suppressed bulb formation in *Lilium longiflorum* • Produced tall, high-quality shoots in azalea shoot tip cultures
Continuous light	• Reduced shoot length and quality of *Rhododendron* sp. shoot tip cultures • Shoot formation was enhanced in shoot tip cultures of *Pelargonium* and leaf segments of *Peperomia scandens* • Shoot formation was enhanced in *Anthurium andraeanum* cultures
Root Formation	
Photoperiod	**Effect on culture**
Continuous darkness	• Root formation was greatest on stem explants of 'Catawbiense Album' rhododendron • Promoted root formation of 'Pink Pearl' rhododendron • Induced a higher percentage of rooted apple plantlets • Inhibited root formation in leaf discs of *Prunus mahaleb*
16 h / 8 h light/dark	• 100% rooting of 'Delicious' apple by placing shoot cultures in dark for a week and then moving them to 16 hr photoperiod
Continuous light	• Prevented root formation of 'Pink Pearl' rhododendron

DE CAPITE (1955) tested plant tissue cultures in natural light, artificial light and darkness. Light intensity was also tested. Optimal growth of *Parthenocissus tricuspidata, Helianthus annuus* and *Daucus carota* was achieved in continuous artificial light. Tissues grown in natural light or darkness grew less than those in continuous artificial light. The effects of plants grown under continuous artificial light at three different intensities were investigated, namely 150, 350 and 750 ft.-c., which is approximately 21.9, 51.1 and 109.5 µmol/m^2/s, respectively (values converted using a factor of 0.146 for cool white fluorescent lamps; THIMIJAN & HEINS, 1983). The results showed that optimum growth was obtained at ± 51.1 µmol/m^2/s (DE CAPITE, 1955).

LESHEM *et al.* (1982) who worked on *Lilium longiflorum* and KROMER (1989) who worked on *Muscari racemosum* both found that light affected bulblet growth and the number of roots produced. LESHEM *et al.* (1982) however, also found that light affected the number of bulblets produced per explant. KROMER (1989) reported that in the light and in the dark a comparative number of explants formed roots, but the explants grown in the light produced a greater number of roots than those in the dark. KROMER (1989) also observed there was an essential difference in the number of emerging leaves per bulblet. In the light the number of leaves was 20% higher than in the dark. This finding was confirmed by LESHEM *et al.* (1982) who found that bulblets cultured in the light bore many leaves, while those in the dark bore fewer leaves.

LESHEM *et al.* (1982) found that bulblets in the light were noticeably different from the bulblets developing in the dark. Scale sections growing in the light turned green and so did the bulblets developing on them. More bulblets developed in the light but they were smaller than those produced in the dark.

In the study carried out by KROMER (1989) it was noted that light slightly increased the percentage of regenerating bulblets but did not influence the number of bulblets produced per explant. The results obtained by LESHEM *et al.* (1982) are not in

agreement with KROMER's findings. LESHEM *et al.* (1982) found no difference between effects of light and dark in the percentage of scales regenerating bulblets although there was a higher number of bulblets produced per explant in the light.

GUDE and DIJKEMA (1992) found that light treatments had no effect on the number of newly formed bulbs in *Hyacinth*. These findings agreed with both LESHEM *et al.* (1982) and KROMER (1989). However, the appearance of the bulbs was greatly affected by the light treatment. Dark-treated bulbs were relatively large, white and undifferentiated as only bulb scales were visible. Bulbs treated with blue light were small but were the most differentiated as the outer bulb scales showed leaf-like structures, the sprouts were well developed and the leaves were green. White-light-treated bulbs showed characteristics similar to blue-light but less pronounced. Lastly, bulbs under red light were a little larger than blue-light-treated bulbs but were less differentiated.

VARSHNEY *et al.* (2000) developed a protocol for the *in vitro* mass production of Asiatic lily hybrids. Previous research showed that the effect of photoperiod on bulblet formation varied with various *Lilium* species especially *L. longiflorum, L. speciosum, L. auratum* and *L. rubellum*. Bulb formation and growth was stimulated by continuous darkness in some species, while in other species it was observed that light significantly affected bulblet growth. Bulblets grown in the light were small and had many leaves while those grown in the dark were large and had few leaves (LESHEM *et al.,* 1982). NIIMI *et al.* (1999) found that there was an increase in the percentage of regenerating bulb scales and the number of bulblets per scale when the cultures were grown in the light. TAKAYAMA and MISAWA (1983) and MAESATO *et al.* (1994) found that continuous light was optimal for bulblet formation for *L. speciosum, L. auratum* and *L. japonicum*. VARSHNEY *et al.* (2000) reported that continuous light was not essential for growth and bulblet multiplication of the Asiatic lily hybrids. The number, size and weight of bulblets increased under a 16 h photoperiod for both cultivars compared to 24 h light and complete darkness.

"Light quality refers to the spectral wavelength of the emitting light. Different kinds of light sources emit light over many wavelengths at different ratios", ECONOMOU and READ (1987). Fluorescent tubes are the main light sources used for *in vitro* cultures, even though different spectral emission characteristics exist among them (ECONOMOU and READ, 1987).

Table 3.2: Summary of results showing the effect of light quality on callus growth and development, shoot formation and root formation (ECONOMOU and READ, 1987).

Callus Growth and Development	
Light quality	**Effect on culture**
Near-ultraviolet (360-450 nm)	• Inhibited callus growth in embryo cultures of *Pseudotsuga menziesii* • Inhibited callus growth and shoot initiation in tobacco callus cultures • No effect on adventitious bud formation in callus from pine embryos • Inhibited callus growth in pine embryos
Blue (450-492 nm)	• Stimulated growth and shoot formation in tobacco callus • Increased the fresh weight of the tissues in *Pelargonium zonale* pith callus • Suppressed callus growth of *Haplopappus gracilis*
Red (647-770 nm)	• Enhanced adventitious bud formation in callus of pine embryo cultures • Did not stimulate shoot initiation in tobacco callus • Suppressed callus growth in *Haplopappus gracilis* • Induced shoot initiation in callus of *Brassica oleracea*
Far-red (770-800 nm)	• Did not stimulate shoot initiation in tobacco callus • Promoted callus growth in *Haplopappus gracilis* • No effect on callus of *Brassica oleracea*
Shoot formation in organ cultures	
Light quality	**Effect on culture**
Blue (450-492 nm)	• No effect on adventitious bud formation of *Pseudotsuga menziesii* embryo cultures
Red (647-770 nm)	• Enhanced adventitious bud formation of *Pseudotsuga menziesii* embryo cultures • Promoted callus growth in embryo cultures of *Pseudotsuga menziesii*

	• Produced more, longer and high quality microshoots in azalea • Produced the greatest number of shoots on leaf discs excised from *Petunia hybrida* plants • Increased the number of shoots formed from *Lactuca sativa* cotyledons • Promoted shoot bud development in root segments of *Convolvulus arvensis*
Root Formation	
Light quality	**Effect on culture**
Near-ultraviolet (360-450 nm)	• Produced more roots in leaf discs of *Prunus mahaleb* when white light was supplemented with near-ultraviolet
Red (647-770 nm)	• Induced root formation in cultures of *Helianthus tuberosus* and seedling nodes of *Eucalyptus ficifloia* • Microcuttings of azalea produced more and heavier roots • *Petunia hybrida* microcuttings grown under white light and then red light, produced more and heavier roots

When studying the effect of light quality and photoperiod on morphogenesis of potato plants *in vitro*, AKSENOVA *et al.* (1994) found that there were pronounced differences in appearance. When stem cuttings of potato plants were placed on medium under red light with a long day photoperiod, the plants were thin, long, had very small leaves and produced no or only a few microtubers. When placed in blue light with a long photoperiod, plants remained short, thick, had large and well developed leaves. They also produced a significant number of microtubers. Cultures placed under the same wavelengths but with a short photoperiod of 10 h, had only slight changes in red light, while in blue light there was a significant lengthening of plants (AKSENOVA *et al.,* 1994).

APPELGREN (1991) found the same results when he researched the effects of light quality on stem elongation of *Pelargonium in vitro*. Shoot cultures placed in red light resulted in the greatest total plant height and stem elongation compared to the results obtained from blue and white light. Blue light actually strongly inhibited stem elongation. Therefore, APPELGREN (1991) reported that by giving the right light conditions shoot elongation can be obtained in *Pelargonium in vitro* and this allows for easy separation of nodal cuttings and axillary buds for multiplication purposes.

When studying light quality and control of shoot length in woody ornamental plants (*Potentilla*, *Rhododendron* 'Lee's Dark Purple', *Spiraea* and *Vitis vinifera*), NORTON *et al*. (1988) found that explants under red light produced longer shoots than under white light. They also discovered that the length of shoots was less when explants were placed under blue light compared to red and white light.

MICHALCZUK and MICHALCZUK (2000) looked at the regeneration rate and plantlet development in *Petunia* 'Revolution' under different light qualities. Results summarized in Table 3.3.

Table 3.3: Summary of results showing the effect of different light qualities on *Petunia* (MICHALCZUK & MICHALCZUK, 2000).

Light Quality	Effect on *Petunia* shoots
Low intensity white (390-760)	• Regenerated shoots with high efficiency • Shoots were short, pale green and had small leaves
High intensity white	• Inhibited shoot regeneration • Promoted the formation of purple-green callus
Blue (450-492 nm)	• Explants regenerated shoots with very high efficiency (100%) • Shoots less abundant but longer and had smaller leaves compared to shoots under green light
Red (647-770 nm)	• Explants regenerated shoots with very high efficiency (100%) • Few shoots regenerated from each explant • Shoots were brittle, pale green, vitrified and leaves curled.
Green (492-550 nm)	• Regeneration rate of shoots was lower (90%) • Produced numerous shoots from a single explant • Shoots were long, dark green and had well developed leaf blades

MICHALCZUK and MICHALCZUK (2000) also found that light quality affected the morphology and proliferation rate of shoots. The highest number of adventitious

shoots were produced under green and red light while the lowest was produced when explants were placed under blue light. Adventitious shoots were obtained under all monochromatic light. The shoots under monochromatic light were longer than the shoots obtained under white light but only for blue light was the difference statistically significant. These results contradict findings by NORTON *et al.* (1988), APPELGREN (1991) and AKSENOVA *et al.* (1994) who found that cultures under blue light inhibited shoot elongation and increased shoot diameter compared to white light illumination.

Various light treatments: blue, green, yellow, red, far-red and UV irradiation, were tested to determine the effect of light qualities on morphogenesis of a few ornamental bulbous plants namely; *Tulipa, Hyacinthus, Galanthus, Cyclamen* and *Freesia* (BACH *et al.,* 2000; BACH & PAWLOWSKA, 2006). The results are presented in Table 3.4 and Table 3.5 below.

Table 3.4: Effect of light quality on somatic embryogenesis of ornamental bulbous plants (BACH *et al.,* 2000; BACH & PAWLOWSKA, 2006).

Light quality	Effect on Somatic Embryogenesis
Blue (450-492 nm)	• Inhibited the development of embryogenic callus in tulip and snowdrop cultures • Stimulated direct development of globular somatic embryos in tulips • Decreased the total number of embryos • Inhibited maturation of somatic embryos of tulips, *Hyacinth* and *Freesia* • Inhibited *Cyclamen* embryo development • Stimulated proliferation of embryogenic callus in *Lilium* cultures
Green (492-550 nm)	• Inhibited the development of tulip callus cultures • Stimulated direct development of globular somatic embryos in tulip
Yellow (550-588 nm)	• Promoted embryogenic callus initiation and growth in cultures of *Cyclamen, Freesia* and *Hyacinth*
White (390-760 nm)	• Inhibited the development of embryogenic callus in tulip and snowdrop cultures

	• Stimulated direct development of globular somatic embryos in tulip
Red (647-770 nm)	• Promoted embryogenic callus initiation and growth in cultures of *Cyclamen, Freesia* and *Hyacinth* • Improved the maturation of torpedo stage somatic embryos in tulip, *Hyacinth* and snowdrop
UV irradiation (360-450 nm)	• Inhibited the development of tulip callus • Stimulated the direct development of globular somatic embryos in tulip • Inhibited maturation of somatic embryos of *Hyacinth* and *Freesia*
Darkness	• Promoted embryogenic callus initiation and growth in cultures of *Cyclamen, Freesia* and *Hyacinth* • Stimulated direct development of globular somatic embryos

Table 3.5: Summary of results showing the effect of light qualities on organogenesis of ornamental bulbous plants (BACH *et al.,* 2000; BACH & PAWLOWSKA, 2006).

Light quality	Effect on Organogenesis
Blue (450-492 nm)	• Produced small bulbs in *Hyacinth* and tulip • Stimulated adventitious shoot and bud growth in *Hyacinthus, Freesia* and *Cylclamen* • Had a positive effect on the quality of *Lilium* adventitious bulbs • Increased the number of *Hyacinth* adventitious plants • Reduced the number of adventitious *Hyacinth* bulbs • Inhibited the maturation of adventitious bulbs in *Hyacinth*
Green (492-550 nm)	• Stimulated adventitious shoot and bud growth in *Hyacinthus, Freesia* and *Cylclamen* • Had a positive effect on the quality of *Lilium* adventitious bulbs
Yellow (550-588 nm)	• Produced the highest number of bulbs in *Hyacinthus* and tulip. Bulbs were mature and well-developed • Produced adventitious bulbs in *Hyacinthus* and *Freesia*
Red (647-770 nm)	• Produced the highest number of bulbs in *Hyacinthus* and tulip. Bulbs were mature and well-developed • Increased plant elongation in *Hyacinth, Cyclamen,*

	Freesia, tulip and snowdrop • Induced bulb dormancy in *Hyacinth* • Produced adventitious bulbs in *Hyacinthus* and *Freesia*
Far-red (770-800 nm)	• Inhibited the development of plantlets in *Hyacinthus, Freesia* and *Cylclamen* • Induced bulb dormancy in *Hyacinth*
UV irradiation (360-450 nm)	• Produced small bulbs in *Hyacinth* and tulip • Reduced the number of adventitious *Hyacinth* bulbs • Inhibited regeneration of adventitious *Hyacinth* shoots
Darkness	• Produced the highest number of bulbs in *Hyacinthus* and tulip. Bulbs were mature and well-developed • Increased plant length in *Hyacinth* • Inhibited the development of plantlets in *Hyacinthus, Freesia* and *Cylclamen*

Callus of *Actinidia deliciosa* cv Hayward was cultured under blue, red and white light as MULEO and MORINI (1990) were testing the effect of light quality on shoot regeneration from callus. It was found that callus grown in either complete darkness or under white light, produced no shoots. Red light produced the highest number of shoots. The time required for regeneration of new organs was much shorter under red light than that of blue light (15 days compared to 25-28 days respectively). Roots were regenerated under all light treatments, however, the time taken to root was quickest under red light. In addition red light produced the highest number of roots. MULEO and MORINI (1990) therefore, showed that light quality has a great influence on shoot induction.

D'ONOFRIO *et al.,* (1998) examined the effect of light quality on somatic embryogenesis on quince leaves. D'ONOFRIO *et al.* (1998) evaluated the effect of light quality by determining the total number of somatic embryos on the leaf lamina. Embryogenic leaves were observed in red, red plus blue, and white light treatments with percentages decreasing statistically from red to white. The highest level of embryogenesis was always observed in the red light treatment. Blue light treatments caused a greater reduction in the embryogenic response. This is in accordance with BACH *et al.* (2000) and BACH and PAWLOWSKA (2006) who found that blue light

decreased the total number of embryos, inhibited maturation of somatic embryos of tulips, *Hyacinth* and *Freesia* as well as inhibited *Cyclamen* embryo development.

Both light quality and light intensity were studied by MICHLER and LINEBERGER (1987) to determine the effect of light on somatic embryo development in carrot suspension cultures. With regards to light quality, the highest number of embryoids were formed in the dark or under red and green light. Cultures under red light produced multiple cotyledons and orange pigmented radicles. After extended time in culture, carrot cells in blue light produced secondary embryoids. When MICHLER and LINEBERGER (1987) studied light intensity, it was discovered that the highest intensity white and blue light treatments and low intensity red light were inhibitory to growth and somatic embryogenesis. Lastly, different intensities of green light had no affect on embryoid production. From this experiment, MICHLER and LINEBERGER (1987) concluded that both red and blue light may be used to manipulate carrot cell cultures to optimize growth.

In vitro cultures of *Azorina vidalii* were established so that DA SILVA and DEBERGH (1997) could study the effect of light quality on morphogenesis. They tested the influence of high and low ratios of blue/red light (2.3 and 0.9) and red/far red light (1.1 and 0.6) on shoot cultures. Maximum plant length and internode length were obtained from cultures grown under a low ratio of red/far-red light. Blue light reduced plant height because it produced shorter internodes. The highest number of axillary shoots were produced under a high level of red/far-red light, while low levels of red/far-red light produced the least number of axillary shoots. The results obtained from this study are in agreement with the data reported by APPELGREN (1991) for *Pelargonium*, in which blue light strongly inhibited stem elongation.

Both GABRYSZEWSKA and RUDNICKI (1997) and GABARKIEWICZ *et al.* (1997) examined the effects of light quality on the growth and development of shoots and roots. GABRYSZEWSKA and RUDNICKI (1997) studied *Ficus benjamina* while GABARKIEWICZ *et al.* (1997) studied *Dieffenbachia* cv Compacta.

GABRYSZEWSKA and RUDNICKI (1997) found that the highest number of axillary shoots and most leaves were produced under red light but on media with iP, while the longest shoots were produced under red light on media with either no growth regulators or on media containing IAA. All light treatments produced roots. However, red light with IAA in the media had an increased number of roots but white light with auxin free media formed the longest roots. GABARKIEWICZ *et al.* (1997) found that the highest root numbers were produced under red and green light with no iP while the longest roots formed on media with iP under white light supplemented with UV. White light and media with iP produced the highest number of shoots however, complete darkness and without iP produced the longest shoots. These results indicate that shoot and root growth and development can be controlled by light quality. As well as showing the presence of interactions between the growth of the plants, exogenous cytokinin and light quality.

The optimum light intensity needed by cultures differs with the stages of tissue culture, explant type and plant species. Shoot growth and production are influenced by light illuminance as well as the process of rhizogenesis. According to ECONOMOU and READ (1987) root formation may be affected directly or indirectly by light intensity. Light can be applied either to the mother cultures during shoot production and have an after-effect on root formation in the next stage of tissue culture (indirect effect) or directly during the rooting process.

HAMMERSCHLAG (1978) looked at the influence of light intensity on growth of *Geranium* callus. All explants formed tissue callus under each treatment but no shoot or root formation was observed. Callus appearance did however differ. ‘Kleine Liebling’ and ‘Sprinter’ produced green and moderately hard callus under 20 µmol/m^2/s, yellow and friable callus under 100 µmol/m^2/s and yellow and hard callus under 200 µmol/m^2/s. ‘Sincerity’ callus was yellow and hard under all light intensities. It was noted that ‘Kleine Liebling’ callus grew greatest at 100 µmol/m^2/s, while callus growth of ‘Sprinter’ and ‘Sincerity’ was significantly greater at 20 µmol/m^2/s and 100 µmol/m^2/s compared to growth at 200 µmol/m^2/s. All tissues

grew poorly at 200 µmol/m^2/s. It has been reported that light intensity can be inhibitory or stimulatory to callus growth and the results from HAMMERSCHLAG (1978) showed just that. HAMMERSCHLAG (1978) also showed that there are cultivar specificities in the response of tissue cultures to light intensity.

SEIBERT *et al*. (1975) using tobacco callus showed that both wavelength and intensity are important. They also proved that intensity can be inhibitory or stimulatory to callus growth. Near ultraviolet light (371 nm) was found to stimulate, at low irradiances (0.024 mW/cm^2), or inhibit callus growth and shoot initiation, when irradiance was higher than 0.15 mW/cm^2. Blue light was found to also stimulate growth and shoot production but only at a higher intensity than near UV light.

From the above information it can be clearly seen that growth, morphology and differentiation of *in vitro* plantlets are affected by light. The data also demonstrates that the influence of light is not only species specific but also genotypic and even explant specific.

3.2 MATERIALS AND METHODS

Leaf explants (1 cm) were excised from decontaminated leaf material of *E. zambesiaca* plantlets grown *in vitro*. These explants (5) were transferred to culture bottles (6 cm diameter, 10 cm high) containing 40 ml solid MURASHIGE and SKOOG medium (1962). The medium was supplemented with 100 mg/L myo-inositol, 30 g/L sucrose and solidified with 8 g/L agar. No hormones were added. The pH of the medium was adjusted to 5.8 with diluted KOH or NaOH before autoclaving at 121 ℃ and 103 kPa for 20 min.

The cultures were stored in Convirons at 20 ℃ with varying photoperiods. The photoperiods tested were continuous light, continuous dark, 16 hours light / 8 hours dark and 8 hours light / 16 hours dark. The Convirons contained Osram L58W/640 cool white fluorescent bulbs and the light intensity range in the growth chambers was 70 to 90 µmol/m^2/s. After 3 months the bulblets were taken out of the culture jars and

the leaves and roots were removed. The bulblets were weighed and bulb diameter measured.

Forty replicates were used per treatment. Data collected were subjected to one-way analysis of variance (ANOVA). Where there is significant difference (p=0.05), the means were separated using Duncan Multiple Range Test (DMRT). Data analysis was done using SPSS version 15.0.

3.3 RESULTS

After two weeks, small white protuberances appeared on the edges of the leaf explants. This was the first sign of developing bulblets. Bulblets turned green and after a further two months were a good size (3 to 4 mm) and produced leaves. Therefore, successful bulblet induction was achieved.

Figure 3.1 shows the mean number of bulbs produced per explant as well as the mean bulb weight and mean bulb diameter. Explants in continuous darkness produced no bulblets while the remaining treatments produced bulblets. From Figure 3.1 it can be seen there is no significant difference between the photoperiods other than for continuous darkness.

In order to determine the optimum treatment the percent bulb induction was converted to a proportion and multiplied by the bulb mass to enable a more consistent ranking of treatments (ASCOUGH, 2008). Table 3.6 shows the ranked photoperiods and it can be seen that 8 h photoperiod was the optimum treatment.

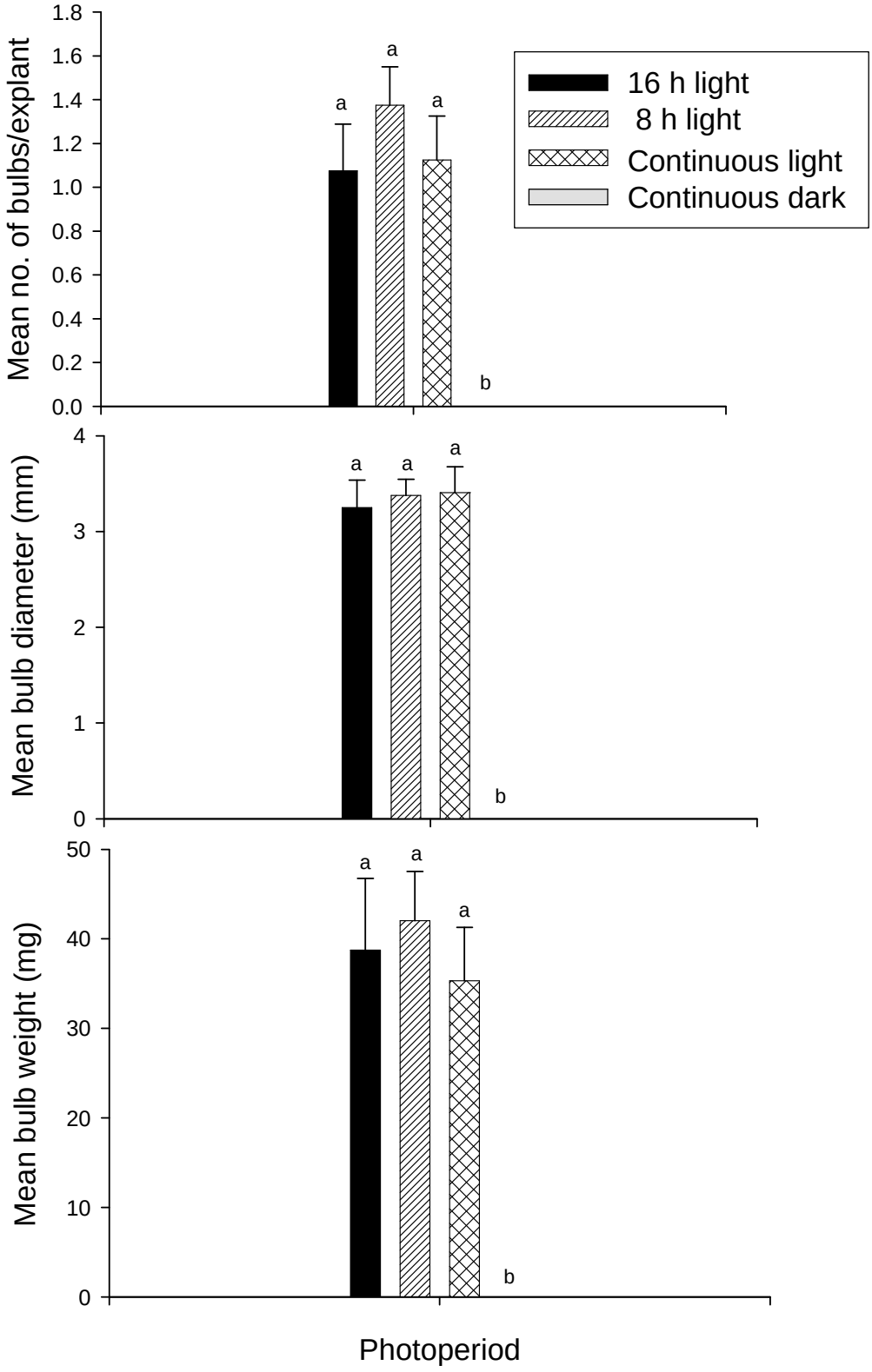


Figure 3.1: Bulblet induction in leaf explants from *E. zambesiaca* tested under four photoperiods. Bars bearing different letters are significantly different, P ≤ 0.05.

Table 3.6: Photoperiods as ranked according to the product which is the proportion bulblet induction multiplied by mean bulb weight. The product shows the optimum treatment.

Photoperiod	Product value
8 h light / 16 h dark	34.69
Continuous light	27.37
16 h light / 8 h dark	16.47
Continuous dark	0

3.4 DISCUSSION

Light is one of the most important environmental factors in tissue culture and the effect it has on explants, can vary greatly. It has been reported that there are three categories of plant response to photoperiod with respect to storage organ formation. These are: induction of storage organs inhibited by darkness, induction of storage organs promoted by darkness and lastly, induction of storage organs by both light and darkness (NIIMI & ONOZAWA, 1979; KIM *et al.,* 1981; STEINITZ & YAHEL, 1982; JACOBS *et al.,* 1992; SLABBERT & NIEDERWIESER, 1999 and ASCOUGH, 2008).

When looking at the results obtained from *E. zambesiaca* for the varying photoperiods, it can be seen that one of the three plant response categories was fulfilled. Continuous darkness completely inhibited bulblet induction. The leaf segments were achlorophyllous and no response or differentiation was observed.

The remaining photoperiods all induced bulblet formation. 8 h photoperiod produced the most number of bulblets per explant (1.4) as well as the heaviest bulbs (42.10 mg) (Figure 3.1). With regards to mean bulb diameter, continuous light had the best results (3.41 mm). From the one-way ANOVA and DMRT it was shown that there were no significant differences between the three photoperiods 16 h/8 h, 8 h/16 h and continuous light, however, they were significantly different from continuous

darkness. Therefore, by looking at Table 3.6 which shows the treatments ranked according to their product, it was clear that 8 h photoperiod was the optimum photoperiod for bulblet induction of *E. zambesiaca* with a product value of 34.69.

The results obtained from these experiments are in disagreement with the data obtained by ECONOMOU and READ (1987). ECONOMOU and READ (1987) found that continuous darkness promoted bulblet regeneration and production in *Hyacinthus orientalis* bulb scale segments as well as increased bulb number and size in scale segments of *Lilium longiflorum*. They showed that 16 h photoperiod suppressed bulb formation in *Lilium longiflorum*, while continuous light reduced shoot length and quality of *Rhododendron* sp. shoot tip cultures. From the *E. zambesiaca* results it was shown that although continuous darkness completely inhibited bulblet initiation, a short photoperiod of light was needed to induce bulblets.

DE CAPITE (1955), LESHEM *et al.* (1982) and KROMER (1989) all obtained growth in both light and dark conditions, however, explants grown in the dark did not grow as well and produced fewer bulbs. Even though growth was reduced in continuous darkness, the explants did respond. This again contradicts our results. It is possible that the results obtained for *E. zambesiaca* differ from all the previous work carried out, due to the type of explant used or it could be due to the specific plant and species used. MULEO and MORINI (1990) believe that light is a necessary factor in *in vitro* tissue culture but its true importance has not yet been recognized. Further studies on light quality and light intensity on *E. zambesiaca* need to be investigated to optimize the true potential of light.

The culture bottles containing the *E. zambesiaca* leaf explants were grown in growth chambers with Osram L58W/640 cool white fluorescent bulbs. Ordinary cool white fluorescent bulbs have a wavelength range of 400 to 450 nm. This places cool white light on the light spectrum closer to blue light (450-492 nm) and green light (492-550 nm) compared to red light (647-770 nm) and far-red light (770-800 nm). BACH *et al.* (2000) and BACH and PAWLOWSKA (2006) found that blue light produced small

bulbs in *Hyacinth* and tulip as well as stimulated adventitious shoot and bud growth in *Hyacinthus, Freesia* and *Cylclamen*. Bulblets formed on *E. zambesiaca* explants grown under cool white light. Although light quality was not tested, because the wavelength of cool white light is known, the results from *E. zambesiaca* can be included in the white and blue light range.

AKSENOVA *et al.* (1994) and APPELGREN (1991) found the same results for potato plants and *Pelargonium* with respect to light quality. When potato plants were grown under blue light with a long photoperiod, plants remained short, thick, and had large and well developed leaves. Blue light strongly inhibited stem elongation in *Pelargonium* plants. MICHALCZUK and MICHALCZUK (2000) reported the highest number of adventitious shoots were produced under green and red light while the lowest was produced when explants were placed under blue light. Blue light had an inhibitory effect on the number and maturation of adventitious *Hyacinth* bulbs (BACH *et al.,* 2000; BACH & PAWLOWSKA, 2006). The above literature is contradictory to explant response to blue light but the results obtained from the experiments on *E. zambesiaca* showed that bulblet induction and development was promoted in the white and blue light range.

The optimum light intensity needed by cultures of various organs and tissues differs among the stages of micropropagation, explant type and plant species (ECONOMOU & READ, 1987). The light intensity of the growth chambers for experiments carried out on *E. zambesiaca* had a range of 70 to 90 µmol/m^2/s. It has been reported that low level white light has an intensity of 19 to 21 µmol/m^2/s, while high level white light has an intensity of approximately 100 µmol/m^2/s (MICHALCZUK & MICHALCZUK, 2000). Leaf explants of *Petunia* regenerated shoots under dim white light with high efficiency, while under higher level white light, shoot regeneration was inhibited (MICHALCZUK & MICHALCZUK, 2000). *E. zambesiaca* produced bulblets under fairly high light intensities and so this contradicts data by MICHALCZUK and MICHALCZUK (2000).

Gloxinia shoot tip cultures were largest and had the greatest leaf size when grown under 60 µmol/m^2/s, while when cultures were maintained at a light intensity of 200 µmol/m^2/s, shoot growth was inhibited (ECONOMOU & READ, 1987). Number, length and quality of new shoots of *Rhododendron* sp. was increased when shoot explants were grown under a light intensity of 30 and 75 µmol/m^2/s, compared to 10 µmol/m^2/s illuminance (ECONOMOU & READ, 1987). From the results obtained in the *E. zambesiaca* experiments and from the data obtained from other authors, it can be seen that a relatively high light intensity (60 to 100 µmol/m^2/s) is permissive for shoot and bulblet growth.

Light plays an essential role in the tissue culture environment influencing many responses of the cultured plants. The effect of light on *in vitro* plants, however, varies with the type of explant used as well as the plant species.

3.5 SUMMARY

- 8 h photoperiod produced the most bulblets per explant
- 8 h photoperiod produced the heaviest bulbs
- Continuous light produced the bulbs with the largest diameter
- The 8 h photoperiod had the highest product value, therefore making it the optimum treatment

Chapter 4
CARBOHYDRATES

4.1 INTRODUCTION

The *in vitro* culture of cells, tissues and organs requires the addition of a carbon source in the medium, since very few cultures are autotrophic. Sucrose has stimulated bulb growth in bulbous plants such as *Allium*, *Gladiolus* and *Lilium*. The absence of sucrose in the growth media results in a lack of differentiation which subsequently causes a lack of storage organ formation in some species (DE BRUYN *et al.,* 1992). Generally, the optimum sucrose concentrations are between 30 and 60 g/L. This may vary among plant species with some plants needing higher or lower concentrations. The increase in sucrose concentration does not necessarily increase the number of bulblets produced but rather increases bulblet size or may stimulate root formation (SLABBERT & NIEDERWIESER, 1999).

BACH *et al.* (1992b) and STAIKIDOU *et al.* (2005) tested the effects of various carbohydrates on bulblet formation of *Hyacinthus orientalis* and *Narcissus* cultivars St. Keverne and Hawera. Both found sucrose to give the best results. Concentration and kind of carbohydrate strongly affected shoot and bulblet formation. Media with glucose or sucrose gave better results than medium containing fructose. A higher number of bulbs formed on media with 30 g/L carbohydrate compared to media with 60 g/L carbohydrate. Bulblets that developed on medium with a concentration of 60 g/L sucrose or glucose were more mature than the bulblets that formed on medium with fructose or with 30 g/L sucrose or glucose (BACH *et al.,* 1992; BOONEKAMP, 1997). STAIKIDOU *et al.* (2005) found that when increasing the sucrose concentration from 30 g/L to 60 g/L there was no improvement in shoot multiplication, however, bulblet number and weight increased. When shoot clumps of *Narcissus* cv St. Keverne were cultured on media with fructose, severe leaf senescence occurred but growth and leaf initiation continued. The lowest concentration (4.7 g/L) of fructose

increased bulblet weight in cv St. Keverne more than glucose, however, cv St. Keverne produced bulbs with the greatest weight at the highest concentration (37.9 g/L) of sucrose. Increasing the carbohydrate concentration from 19.0 to 37.9 g/L increased bulblet weight of Hawera, no matter which carbohydrate was used.

Bulb growth in lily regenerated *in vitro* was studied by LANGENS-GERRITS *et al.* (1997) and LANGENS-GERRITS *et al.* (2003). During *in vitro* culture, growth of bulblets depended on sucrose concentration. Bulblet dry weight doubled or tripled when the sucrose concentration doubled or tripled. Total carbohydrate and starch content of bulblets increased with sucrose concentration (LANGENS-GERRITS *et al.,* 1997). LANGENS-GERRITS *et al.* (2003) tested various sugars (glucose, fructose, mannose and sucrose). None of the sugars, nor a combination of glucose and fructose exceeded sucrose in promoting the growth of bulblets. The weight per bulblet increased with increasing sucrose concentration.

TAKAYAMA and MISAWA (1980) studied the effects of sucrose concentration on differentiation and scale leaf formation on *Lilium auratum*. The highest percentage of scale leaf formation was obtained when bulbscales were grown on media with 30 g/L sucrose. Medium with 90 g/L sucrose was inhibitory.

Shoot multiplication of two rose cultivars (*Rosa* cvs Iceberg and Peace) was highest on medium with 40 g/L sucrose while a concentration of 10 g/L sucrose was limiting. Shoot growth was greatly affected at lower concentrations of sucrose and the incidence of vitrification increased. Vitrification was rare when explants were grown on media with 40 g/L sucrose, however, large amounts of callus formed. LANGFORD and WAINWRIGHT (1988), therefore suggest that a sucrose concentration of 20 or 30 g/L is optimal for the healthy growth of rose shoots *in vitro*.

TAYLOR and VAN STADEN (2001e) reported that changing the sucrose concentration in the initiation medium resulted in a significant change in the number of shoots produced as well as average mass of the plantlets. Shoot initiation in

Eucomis autumnalis was promoted at a high concentration of sucrose (40 g/L). When the sucrose concentration was lowered the number of shoots was reduced. A high (40 g/L) sucrose concentration increased the average mass of each plantlet, while low (10 g/L) sucrose levels significantly decreased the mass of the plantlets (TAYLOR & VAN STADEN, 2001e).

VISHNEVETSKY *et al.* (1997) studied *Nerine sarniensis* and observed that bulbs cultured on 60 g/L sucrose medium grew better than those on medium with 30 g/L sucrose. The ratio between bulb and leaf weight was higher with 60 g/L sucrose than with 30 g/L sucrose in the medium. Lastly, medium that contained 90 g/L sucrose inhibited bulb growth and leaf elongation. SLABBERT and NIEDERWIESER (1999) claim that the most important factors influencing *in vitro* bulblet formation of *Lachenalia* are; sucrose concentration, temperature and length of the explant. With regards to sucrose concentration; higher sucrose concentrations (60 g/L) did not influence bulbing, but led to the formation of larger bulbs compared to those produced on 30 g/L sucrose.

BACH & PAWLOWSKA (2006) reported that the development and growth of Hyacinth plantlets can be controlled by balancing different carbohydrates and the type of light. A low or moderate level (30 or 60 g/L) of sucrose produced a high number of bulbs when explants were grown under red or white light or in complete darkness. The highest number of bulbs were formed on a medium with the highest glucose concentration (120 g/L). To produce shoots of hyacinths *in vitro* it was beneficial to use glucose as the carbohydrate source and blue light. Red and far-red light as well as darkness was found to negatively affect the formation of shoots cultured on media with sucrose, while glucose in the same light conditions promoted the induction of shoots (BACH & PAWLOWSKA, 2006).

Higher sucrose concentrations have been reported to promote bulblet formation in tulip (TAEB & ALDERSON, 1990) and to increase bulb weight in hyacinth (BACH,

1992) but elevated sucrose concentrations had no additional beneficial effect on bulbing in *Narcissus* (HUSSEY, 1982b).

CHOW *et al.* (1992) studied how sucrose stimulates bulb formation in *Narcissus in vitro.* It was found that few bulbs formed from shoots (9%) when grown on media with 30 g/L sucrose, but increasing the sucrose concentration to 60 g/L or 90 g/L increased the percentage of shoots forming bulbs to 49% and 71% respectively. When there was no sucrose present in the media, shoots senesced rapidly but no bulbs formed. High sucrose concentration stimulated bulb formation very strongly and bulb diameter increased more than three fold by increasing the sucrose concentration from 30 g/L to 90 g/L. High sucrose concentrations strongly inhibited leaf elongation *in vitro*, however, after 8 weeks of growth in the shade house, the effect was much less pronounced and rapid leaf elongation occurred.

CHOW *et al.* (1992) and STAIKIDOU *et al.* (2005) showed that bulb development by shoot clumps could be stimulated by increasing the sucrose concentration in the culture medium. The results obtained by SANTOS *et al.* (2002) when working on *Narcissus asturiensis* reinforce the suggestion that a high level of sucrose caused the enlargement of bulbs and allowed the accumulation of food reserves.

SELLÉS *et al.* (1997) looked at the effect of sucrose on growth in shoot-clump cultures of *Narcissus confusus* in liquid-shake cultures and their findings were in agreement with CHOW *et al.* (1992). Different concentrations of sucrose (30 g/L to 180 g/L) were tested and it was found that explants showed the best growth when they were cultured with 90 g/L sucrose. Higher concentrations of sucrose led to a decrease in growth. The dry weight/fresh weight ratio was higher at higher sucrose concentrations which was possibly a result of more storage material or a hydric stress of the tissues. These results supported the results obtained by TAKAYAMA and MISAWA (1979) who suggested that the effect of sucrose on bulb growth of *Lilium auratum* could be mediated through changes in osmotic potential.

When the lily hybrid Star Gazer was studied by KUMAR *et al.* (2005), it was discovered that the number of bulblets was higher at 90 than at 60 g/L sucrose. Higher concentrations stimulated the fresh mass of bulblets. MORÁN *et al.* (2003) also found that 90 g/L sucrose produced better results than 60 g/L for both *Cyrtanthus clavatus* and *Cyrtanthus spiralis*. Shoots that were grown on 60 g/L sucrose produced roots and leaves whereas shoots grown on media containing 90 g/L sucrose were the biggest and heaviest. Bulbs from both species of *Cyranthus* that were grown on media supplemented with 60 or 90 g/L sucrose had a 100% regeneration of shoots as well as a greater bulblet regeneration and biomass gain (MORÁN *et al.,* 2003). This was also observed in other bulbous plants such as *Allium* (KELLER, 1992) and *Narcissus* (CHOW *et al.,* 1992). Bulblets grown on 30 g/L sucrose formed a good root system while bulbs grown on 120 g/L did not form leaves or shoots but enlarged in size. The best bulbing treatment for *C. clavatus* and *C. spiralis* was media with 90 g/L sucrose. This is in accordance with results obtained by SELLÉS *et al.* (1997) for *Narcissus confuses*.

ANGULO *et al.* (2003) worked on the *in vitro* production of bulblets of *Cyrtanthus loddigesianus* and *Cyrtanthus speciosus.* The highest biomass gain was observed in liquid media supplemented with 90 g/L sucrose for *C. loddigesianus* and 30 g/L sucrose for *C. speciosus.* With regards to the growth index values, there was no statistically significant difference when shoot clumps of the two species were grown in four sucrose concentrations (30 g/L to 120 g/L). The results for *C. loddigesianus* confirm results for other bulbous plants such as *Narcissus confuses* (SELLÉS *et al.,* 1997) and *Lilium rubellum* (NIIMI *et al.,* 2000). SELLÉS *et al.* (1997) and NIIMI *et al.* (2000) however found that high sucrose concentrations increased biomass gain in bulblets.

A protocol for the *in vitro* mass propagation of Asiatic lily hybrids was developed by VARSHNEY *et al.* (2000) and it was found that the use of sucrose at different concentrations at different stages of bulblet growth is important. At the initiation stage, sucrose (30 g/L) was the optimum concentration for bulblets initiation as the

number of bulblets per explant and leaf formation was highest at this concentration. As the sucrose concentration increased the number of bulblets per explant decreased. This contradicts TAKAYAMA and MISAWA's (1983) work on *Lilium speciosum* and *Lilium auratum* where it was found that 90 g/L sucrose was optimal for bulblet initiation. At the growth stage, increased sucrose concentration increased the Asiatic lily hybrids bulblet growth. For cv Gran Paradiso 60 g/L and 90 g/L sucrose increased bulblet size twofold but the multiplication rate was lower at 90 g/L therefore, 60 g/L sucrose was the optimum concentration for cv Gran Paradiso. For cv Sanciro, 90 g/L sucrose was the optimum. Concentrations of 120 g/L sucrose and higher, did not increase multiplication or bulblet growth. Compared to glucose and fructose, sucrose was the best carbohydrate for the multiplication of bulblets. Therefore, for cv Gran Paradiso and cv Sanciro sucrose at concentrations of 60 g/L and 90 g/L are the optimum sugar treatments.

VAN RENSBURG *et al.* (1988) micropropagated *Ornithogalum maculatum* and found that sucrose concentration had an effect on the formation and growth of shoots and callus. Fewer shoots per explant formed on medium with 10 g/L sucrose, while shoots developed roots on medium with 30 g/L sucrose. 50 to 70 g/L sucrose was the optimum concentration as bulblets with short leaves developed. The percentage of explants forming callus and the size of callus decreased with increasing concentrations of sucrose. Callus on 30 g/L sucrose developed adventitious roots. It has been suggested that with the same auxin/cytokinin ratio, organogenesis varies with the kind and quantity of carbohydrate, the action of which seems to be both metabolic and osmotic (VAN RENSBURG *et al.,* 1988). It is believed that this is reflected in the growth of *O. maculatum* leaf explants grown at different concentrations of sucrose.

The effect of sucrose concentration on the initiation and growth of adventitious buds was studied on six *Lachenalia* genotypes by VAN RENSBURG and VCELAR (1989). The optimum sucrose concentration varied with the different genotypes: 30 g/L sucrose produced the best results for Romaud, Rolina formed more buds on medium

supplemented with 50 g/L sucrose, while Romargo responded well on sucrose at 50 g/L to 90 g/L. The optimum sucrose concentration for Robyn was 10 g/L. 70 g/L yielded the best results for breeding line 75/18/20, while there was no significant difference in numbers on 10 to 90 g/L sucrose for breeding line 78/93/17. In general the higher the sucrose concentration, the shorter the shoots growing on the medium. The different genotypes responded differently to the sucrose concentration range. Therefore, there will be differences in response with different species to different sugar concentrations, let alone different sugars.

There are several methods of inducing somatic embryogenesis without applying plant growth regulators and one of these methods include treatments with high concentrations of sucrose. KURATA *et al.* (1992) induced somatic embryogenesis in carrot cultures by liquid shake culture using a high sucrose (250 g/L) containing medium.

Bulblet growth *in vitro* is an important factor for rapid growth of lily bulblets after planting. Growth, once transferred to soil, depends on bulblet size as larger bulbs grow faster and form stems more frequently. It has been reported that large bulbs can be obtained *in vitro* by culture on medium with a high sucrose concentration (MOHAMED-YASSEEN *et al.,* 1994). MOHAMED-YASSEEN *et al.* (1994) studied the *in vitro* shoot proliferation and production of sets from garlic and shallot. High sugar concentrations *in vitro* increased bulb size in shallot and *Lilium auratum* (TAKAYAMA & MISAWA, 1980). High sugar concentrations were reported by TAKAYAMA and MISAWA (1980) to induce dormancy in *in vitro* bulbs. However, no dormancy was observed by MOHAMED-YASSEEN *et al.* (1994).

Carbohydrates play a significant role in the growth and enhancement of bulb size in tissue cultured plants. However, the type of carbohydrate and concentration may vary with each plant.

4.2 MATERIALS AND METHODS

Plant material from established *in vitro* cultures was used. Leaves from the *in vitro* plants were cut into 1 cm sections and placed in culture bottles (6 cm diameter, 10 cm high) on 40 ml standard MURASHIGE and SKOOG medium (1962), with 100 mg/L myo-inositol and solidified with 8 g/L agar.

Table 4.1: Different carbohydrates tested at different concentrations and in combination with each other.

Carbohydrate	Concentration (g/L)
Control	0
Sucrose	10, 30, 60, 90 and 120
Fructose	10, 30, 60, 90 and 120
Glucose	10, 30, 60, 90 and 120
30 g/L Glucose + Fructose	15 glucose + 15 fructose
60 g/L Glucose + Fructose	30 glucose + 30 fructose
90 g/L Glucose + Fructose	45 glucose + 45 fructose
Glucose:Fructose (1:2)	10 glucose : 20 fructose
Glucose:Fructose (2:1)	20 glucose : 10 fructose

The pH of the medium was adjusted to 5.8 with diluted KOH or NaOH before autoclaving at 121 ℃ and 103 kPa for 20 min. The culture bottles were placed in an environmental chamber (Conviron) with a 16/8 h light/dark regime and a temperature of 20 ℃. The growth chambers contained Osram L58W/640 cool white fluorescent bulbs and had a light intensity range of 70 to 90 µmol/m^2/s. After 3 months, the bulblets were removed, and bulblet mass and diameter were determined.

Forty replicates were used per treatment. Data collected were subjected to one-way analysis of variance (ANOVA). Where there is significant difference (p=0.05), the means were separated using Duncan Multiple Range Test (DMRT). Data analysis was done using SPSS version 15.0.

4.3 RESULTS

Growth was observed after two to three weeks in the environmental chambers with the appearance of white globular shaped protuberances. The white protuberances turned green and developed into bulblets. The number of bulblets produced per leaf explant decreased as the concentration of the carbohydrate increased. Little shoot and bulblet growth was observed at 60 g/L sucrose and fructose (Figure 4.1). Higher concentrations of sucrose, fructose and glucose reduced bulblet growth. At 120 g/L fructose, the leaf explants turned brown and no response was seen. A concentration of 30 g/L fructose gave the best results for all parameters measured; mean number of bulbs per explant, mean bulb diameter and mean bulb weight (Table 4.2).

Both glucose:fructose ratios (1:2 and 2:1) produced good results. Medium supplemented with 30 g/L fructose and 2:1 glucose:fructose both had the highest mean bulb diameter value of 3.39 and 3.36 mm as well as the highest mean bulb weight of 56.61 and 46.27 mg, respectively (Table 4.2).

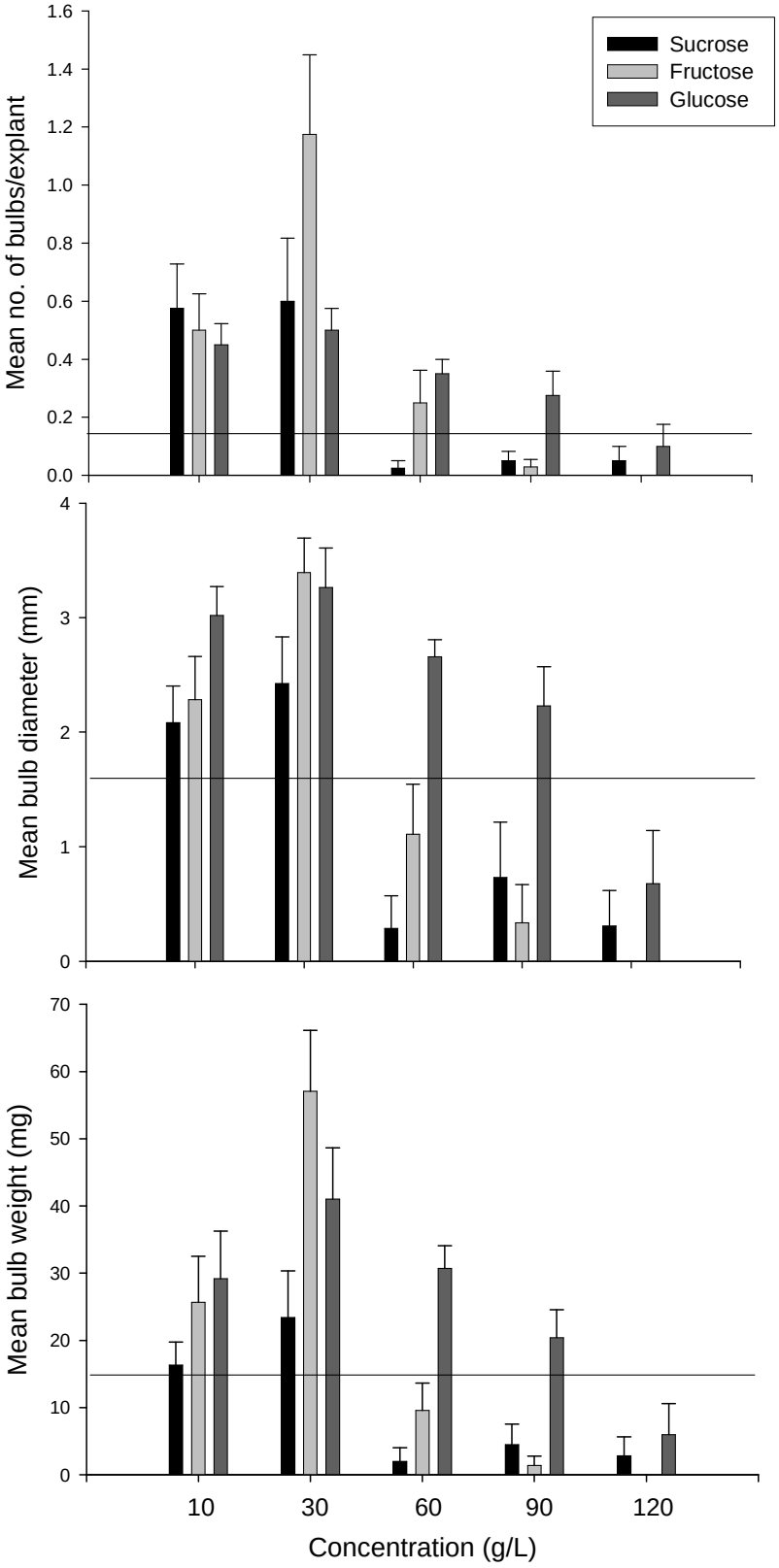


Figure 4.1: Bulblet induction in leaf explants from *E. zambesiaca* grown on media with various carbohydrates at different concentrations. The line indicates the control.

Table 4.2: Results from the different carbohydrate treatments and the significant differences.

Carbohydrate (g/L)	Mean no. of bulbs/explant	Mean bulb diameter (mm)	Mean bulb weight (mg)
Control	0.16±0.07^{cde}	1.69±0.68^{def}	15.50±6.36^{efghi}
10 Sucrose	0.58±0.15^{b}	2.08±0.32^{cdef}	16.31±3.44^{defgh}
30 Sucrose	0.60±0.22^{b}	2.42±0.41^{abcd}	23.40±6.92^{def}
60 Sucrose	0.03±0.03^{de}	0.29±0.29^{gh}	2.00±2.00^{hi}
90 Sucrose	0.05±0.03^{de}	0.73±0.49^{gh}	4.50±3.04^{hi}
120 Sucrose	0.05±0.05^{de}	0.31±0.31^{gh}	2.81±2.81^{hi}
10 Fructose	0.50±0.13^{b}	2.28±0.38^{bcde}	25.64±6.88^{de}
30 Fructose	1.18±0.27^{a}	3.39±0.30^{a}	56.61±9.10^{a}
60 Fructose	0.25±0.11^{bcde}	1.11±0.44^{fg}	9.58±4.04^{fghi}
90 Fructose	0.03±0.03^{de}	0.34±0.34^{gh}	1.38±1.38^{hi}
120 Fructose	0.00±0.00^{e}	0.00±0.00^{h}	0.00±0.00^{i}
10 Glucose	0.45±0.07^{bc}	3.02±0.25^{abc}	29.20±7.04^{cd}
30 Glucose	0.50±0.08^{b}	3.26±0.34^{ab}	40.99±7.66^{bc}
60 Glucose	0.35±0.05^{bcde}	2.66±0.15^{abcd}	30.71±3.36^{cd}
90 Glucose	0.28±0.08^{bcde}	2.23±0.34^{bcde}	20.38±4.20^{defg}
120 Glucose	0.10±0.08^{de}	0.68±0.46^{gh}	5.96±4.63^{ghi}
15 Glucose + 15 Fructose	0.38±0.07^{bcd}	1.83±0.27^{def}	11.88±1.91^{efghi}
30 Glucose + 30 Fructose	0.33±0.06^{bcde}	1.86±0.29^{def}	13.50±2.55^{efghi}
45 Glucose + 45 Fructose	0.25±0.08^{bcde}	1.34±0.42^{efg}	10.83±3.94^{efghi}
Glucose:Fructose (1:2)	0.50±0.05^{b}	2.71±0.14^{abcd}	31.85±3.32^{cd}
Glucose:Fructose (2:1)	0.45±0.07^{bc}	3.36±0.24^{a}	46.27±6.86^{ab}

Mean values in the same column followed by the same letter(s) are not significantly different (p=0.05).

Table 4.3: Different carbohydrates and concentrations of carbohydrates affecting bulblet induction and the product value.

Carbohydrate	Concentration (g/L)	Product value
Control	0	1.94
Sucrose	10	6.93
	30	12.29
	60	0.05
	90	0.23
	120	0.14
Fructose	10	8.97
	30	38.52
	60	2.16
	90	0.03
	120	0.00
Glucose	10	8.76
	30	17.42
	60	10.75
	90	4.58
	120	0.45
Glucose and Fructose	15 + 15	5.34
	30 + 30	4.39
	45 + 45	2.17
Glucose:Fructose	(1:2)	15.13
	(2:1)	20.82

4.4 DISCUSSION

Relatively few studies have been conducted on the effect of different carbohydrate concentrations on bulb formation and growth of bulbous plants. Studies in which the sucrose concentration was varied are few and include those of NIIMI and ONOZAWA (1979) and TAKAYAMA and MISAWA (1979) on *Lilium* species and VAN RENSBURG and VCELAR (1989) on *Lachenalia*.

Sucrose is considered to be the best source of carbon for *in vitro* tissue culture (GEORGE, 1993). Sucrose is normally hydrolysed partially or completely in the

medium into the monosaccharides glucose and fructose, which are taken up by the plant tissues. According to GEORGE (1993) the monosaccharides are taken up partly through active transport and partly through passive permeation.

Fructose was established as the best carbohydrate for *E. zambesiaca*, in particular 30 g/L fructose. There could be a number of reasons for this. The first, being that perhaps *E. zambesiaca* has a different sugar sensing and signaling system. This means that the plant senses or recognizes fructose better than the other carbohydrates. The second could be that when the plant is presented with different carbohydrates such as sucrose, fructose and glucose, it selects/prefers fructose as it does not have to break down sucrose. By selecting fructose the plant eliminates the energy needed to hydrolyze sucrose and provide energy for growth.

BACH *et al.* (1992b) studied the effects of sucrose, fructose and glucose on bulblet formation on *Hyacinthus orientalis* leaf explants. Media supplemented with fructose produced more bulblets than the other carbohydrates. It was also found that more bulblets were produced on media with a concentration of 30 g/L carbohydrate as opposed to a 60 g/L concentration. The results from the experiments carried out on *E. zambesiaca* looking at various carbohydrates, concentrations and combinations of carbohydrates are in agreement with the data obtained by BACH *et al.* (1992b). Most bulblets were produced at 30 g/L fructose.

After 30 g/L fructose, 2:1 glucose:fructose ratio was found to give the next best results. There was no significant difference between 30 g/L fructose and 2:1 gucose:fructose, for both mean bulb diameter (3.39 and 3.36 mm) and mean bulb weight (56.6 and 46.3 mg) respectively. These results contradict those of LANGENS-GERRITS *et al.* (2003) who tested various sugars (glucose, fructose, mannose and sucrose) on lily. None of the sugars, nor a combination of glucose and fructose exceeded sucrose in promoting the growth of bulblets. The weight per bulblet increased with increasing sucrose concentration. This was also found to not be true

with bulblets of *E. zambesiaca* as when the carbohydrate concentration increased, mean bulblet weight decreased.

The third reason why fructose was the best carbohydrate could be due to osmotic potential. 30 g/L fructose has a different osmotic potential than 30 g/L sucrose or 30 g/L glucose. DE PAIVA NETO & OTONI (2003) published a review paper warning scientists against using percentages as reference concentrations to determine the quantities of the different carbohydrates contained in treatments as this leads to conflicting results and conclusions. DE PAIVA NETO & OTONI (2003) stated that when different sugars are added using the reference in w/v or percentage rather than molar concentration, then an osmotic variable is introduced in the treatments and this may mask the explant response. Therefore, molar concentrations should be used as it isolates the osmotic variable influence and in so doing, the response of the explants will be exclusively due to the nutritional factor of the carbon source.

STAIKIDOU *et al.* (2005) found that with the *Narcissus* cv St. Keverne, all monosaccharide media gave much lower shoot clump and bulb weights than sucrose. This could be due to osmolarity, which was 380 m Osm kg^{-1} for sucrose medium but 650 m Osm kg^{-1} for monosaccharide media. However, no detrimental effects on shoot or bulblet weight were seen when cv Hawera was grown on media with monosaccharides. STAIKIDOU *et al.* (2005) suggest that the responses may be related to their origin as cv St. Keverne is derived from *N. pseudonarcissus* which is native to the UK, while cv Hawera shows characters of *N. triandrus* which is native to the Iberian Peninsula. *N. triandrus* is more likely than *N. pseudonarcissus* to be exposed to water stress in its natural environment. *Eucomis zambesiaca* is found occurring from northern South Africa to Malawi. Northern South Africa is generally hot and dry and so *E. zambesiaca* is probably also exposed to water stress like *N. triandrus*. This could explain why *E. zambesiaca* grew well on media supplemented with fructose.

The fourth and final reason could be plant specificity. MEZZETTI *et al.* (1991), states that the capacity of plant tissues to utilize carbohydrates varies between species and even explants. It also depends on the plants ability to absorb, transport and metabolize the carbohydrates. TAYLOR and VAN STADEN (2001e) studied the effect of sugar concentration on *Eucomis autumnalis*. A high concentration of sucrose (40 g/L) promoted shoot initiation and growth. From the experiments carried out on *E. zambesiaca* it was observed that fructose was the best treatment for bulblet induction. Therefore, two different *Eucomis* species responded differently to different carbohydrates. This is in accordance with what MEZZETTI *et al.* (1991) claims.

4.5 SUMMARY

- 30 g/L fructose produced most bulbs per explant
- 30 g/L fructose and glucose:fructose (2:1) yielded the largest mean bulb diameters
- 30 g/L fructose and 2:1 glucose:fructose produced bulbs with the heaviest mean weights

Chapter 5
PLANT HORMONES

5.1 INTRODUCTION

Plant growth regulators or hormones are naturally occurring organic substances that are needed in low concentrations to facilitate essential plant processes. The processes influenced by plant hormones include growth, dormancy, flowering, cell differentiation and fruit ripening (DAVIES, 1987). Environmental factors such as temperature, light and day length interact with plant hormones to cause developmental responses in plants.

Plant growth regulators are the critical media components in determining the developmental pathway of the plant cells, however, there is some considerable difficulty in predicting the effects of these plant growth regulators for tissue culture. This is due to the differences in culture response between species, cultivars and even plants of the same cultivar grown under different conditions. There are five major groups of plant growth regulating compounds; auxin, cytokinin, gibberellin (GA), ethylene and abscisic acid (ABA) (GASPAR *et al.,* 1996). Both auxins and cytokinins are present in all plants at any time and in all the major organs. Auxins and cytokinins are needed for essential developmental processes and no plant can develop in their absence. Gibberellins, ethylene and abscisic acid are widespread in plants and have a number of important roles however, none of these three plant growth regulators are essential although they are important messengers (DAVIES, 1987).

SKOOG and MILLER (1957), while working on the regeneration of tobacco, discovered that the balance between auxins and cytokinins plays a role in the course of organogenesis and morphogenesis. When there is a high level of auxin to

cytokinin then roots develop while with a high level of cytokinin to auxin, then shoots form. When the ratio of cytokinin to auxin is about the same, a callus mass is produced. It is now well established in tissue culture, that by adjusting the auxin:cytokinin ratio, shoots or roots can be induced (HEMPEL, 1979; KRIKORIAN *et al.,* 1987). In some plant species the influence of other growth regulators such as gibberellins and inhibitors are also involved.

Auxins play a role in cell enlargement and elongation and in cultured systems they promote cell division (GASPAR *et al.,* 1996). Auxins stimulate stem growth and root initiation. There are a number of naturally occurring auxins, however, most are not available for use other than indole-3-acetic acid (IAA) and indole-3-butyric acid (IBA). Synthetic auxins are often used in micropropagation as they are more stable. The most commonly available and used are 2,4-dichlorophenoxyacetic acid (2,4-D) and 1-naphthaleneacetic acid (NAA) (DAVIES, 1987; KRIKORIAN *et al.,* 1987).

In tissue culture cytokinins are necessary for plant cell division (STRNAD, 1997). They are also very effective in promoting direct and indirect shoot initiation. A low concentration of cytokinin is often used in the culture medium to induce embryogenic callus. However, there is evidence that suggests that exogenous cytokinins could inhibit embryogenesis in monocotyledons and certain plant genotypes. This could be a result of the presence of endogenous cytokinins. Cytokinins are used to stimulate the growth of axillary buds and reduce apical dominance in shoot cultures (GASPAR *et al.,* 1996). High levels of cytokinin have been found to cause small shoots to form which do not elongate, leaves to have an unusual shape, or induce shoots to become hyperhydric. High concentrations of cytokinins also generally inhibit or delay root formation. Natural cytokinins include N^6-isopentenyladenine (iP) and zeatin. Zeatin is about 10 times more potent than any synthetic cytokinin, however, both zeatin and iP are not commonly used as they are expensive. The synthetic cytokinins most commonly used in tissue culture are kinetin and N^6-benzyladenine (BA), although it is now accepted that BA derivatives occur naturally (GEORGE & SHERRINGTON, 1984; DAVIES, 1987; STRNAD, 1997).

There exists over 87 naturally occurring, structurally related compounds called gibberellins (GAs). Gibberellins are widespread and are ubiquitous in both angiosperms and gymnosperms. They are believed to be synthesized in young tissues of the shoot tips, root tips and also plant embryos. The main effect of GAs in plants is to stimulate stem elongation by stimulating cell division and elongation. Flowering is also stimulated (GASPAR *et al.,* 1996). They are important in determining plant height and fruit set, however, only a few of the GAs are used in tissue culture. The most common and widely available GA is GA_3, gibberellic acid. GA_{4+7} is also used. Both GAs are used as they can inhibit callus formation and auxin-induced adventitious root formation (SMITH, 2000). Gibberellins have been used for stem elongation in cases where the shoots have not elongated naturally, however, in most plants, GAs produce abnormally long shoots with excessively narrow leaves. It has been reported that GAs also control various aspects of seed germination, including the loss of dormancy and the mobilization of endosperm reserves (TAIZ & ZEIGER, 2002). The transition from the juvenile to mature stage, floral initiation, sex determination and fruit set are also all affected by GAs.

Abscisic acid (ABA) is a single compound and occurs naturally in plants. ABA has been found to be present everywhere within the plant. It has been detected in every major organ or living tissue from the root cap to the apical bud. ABA is synthesized in almost all the cells that contain chloroplasts and amyloplasts. As with the other hormones, the response to ABA depends on the concentration within the tissue and on the sensitivity of the tissue to the hormone. ABA is generally thought to play mostly inhibitory roles, however, it has many promoting functions as well (GASPAR *et al.,* 1996). The production of ABA is stimulated by stresses such as water loss and freezing temperatures, therefore, ultimately ABA causes responses that help protect plants against environmental stresses. Application of ABA to leaves can cause stomatal closure but the most common responses to ABA are the inhibition of; stem growth, growth of radicles and germination of seeds. It has been found that ABA causes seed dormancy in some species (ADDICOTT, 1983) but not in others. In

plant tissue culture ABA is used to promote distinct developmental pathways such as somatic embryogenesis (GASPAR *et al.,* 1996). ABA has also been found to be important during maturation and germination of normal somatic embryos.

The last important plant growth regulator is ethylene. Ethylene is unique in that it is a gaseous hormone unlike the rest of the plant hormone compounds. Like abscisic acid it is the only member of its class. It is produced in all higher plants and is produced from methionine in essentially all tissues. Production of ethylene varies with the type of tissue, the plant species and also the stage of development. It is most commonly associated with a response to stress, fruit ripening, leaf abscission, promotes flower senescence and the triple response where, stem elongation is inhibited and stem thickening and horizontal growth increased (TAIZ & ZEIGER, 2002). The use of ethylene in plant tissue culture is not widespread as it presents a problem for *in vitro* propagation. Some plant cultures produce ethylene which if it builds up sufficiently, can inhibit the growth and development of the culture. However, it has been reported to promote shoot and root growth and differentiation as well as adventitious root formation (DAVIES, 1987; GASPAR *et al.,* 1996).

The most commonly used cytokinin in the tissue culture of plants is BA, due to its effectiveness and affordability. It does however have disadvantages as it can induce genetic alteration and abnormal growth in some plants, and has been found to cause hyperhydricity in many species. A derivative of BA, [9G]BA (9-glycosylbenzyladenine), has been reported to cause heterogeneity in growth and inhibition of rooting during acclimatization (BAIRU *et al.,* 2007). Zeatin naturally occurs in plants, however, it is less widely used as it is very expensive. Therefore, much research is being carried out to find an alternative to BA and zeatin.

A possible alternative for BA and zeatin is *meta*-topolin (*m*T) and its derivatives. BAIRU *et al.* (2007) looked at optimizing the micropropagation protocol for *Aloe polyphylla* by using *meta*-topolin instead of BA. At low concentrations, BA produced more shoots however, as the concentration increased, larger numbers of shoots were

obtained from the *m*T treatments. Plants treated with *m*T were superior in quality and quantity compared to those grown on medium supplemented with BA. As the concentration of cytokinin increased, so did the incidence of hyperhydricity although hyperhydricity was most severe in BA treatments. BA-treated plants were also yellowish in colour, had abnormal shoot growth and failed to root. Therefore, due to a higher multiplication rate, high morphogenetic activity and good rooting, BAIRU *et al.* (2007) found that *m*T could possibly be a good replacement for BA and zeatin in tissue culture.

VAN STADEN and DREWES (1994) investigated the effect of BA and its glucosides on adventitious bud formation on *Lachenalia* leaf sections. The four BA derivatives tested were; BA, [3G]BA (3-glycosylbenzyladenine), [7G]BA (7-glycosylbenzyladenine) and [9G]BA (9-glycosylbenzyladenine). Of the four cytokinins tested, BA increased adventitious bud formation in *Lachenalia*. The best results were obtained when the culture medium was supplemented with 4 mg/L BA and 2 mg/L NAA. VAN STADEN and DREWES (1994) believe that the different responses with different cytokinin derivatives may be a function of uptake, transport or metabolism of the applied cytokinin.

Medium supplemented with BA increased the production of embryogenic structures as well as the maturation of somatic embryos in *Freesia hybrida* (BACH, 1992). ULRICH *et al.* (1999) looked at the micropropagation of *Crinum* 'Ellen Bosanquet'. All BA concentrations (1, 2 and 5 mg/L) enhanced shoot formation but the greatest shoot formation was on medium with 5 mg/L BA. NAA had an inhibitory effect on shoot formation as fewer shoots were formed on NAA containing media compared with the control. Explants grown on medium with NAA became enlarged and swollen. No organs or callus developed. In another experiment, tri-scales were cultured on medium supplemented with 8, 16 or 20 mg/L BA. After four months the explants were transferred to hormone free medium for three months. Optimal shoot and bulblet formation was stimulated when explants were initially grown on media with 8 mg/L BA and then transferred to hormone free media.

Rapid propagation of *Hyacinthus orientalis* bulbs was studied by TAKAYAMA *et al.* (1991). Bulb regeneration was stimulated by a combination of 10 mg/L BA and 0.1 mg/L NAA. Low concentrations of BA and either no NAA or high concentrations of NAA did not stimulate bulblet formation. High concentrations of BA inhibited root development while a high concentration of NAA enhanced the development of regenerative callus. When medium was supplemented with only 0.1 mg/L BA, bulblets enlarged in size.

The effect of growth regulators on regeneration of lily bulbs *in vitro* was investigated by DABROWSKI *et al.* (1992). Bulblet formation occurred on explants grown on media containing 0.1 mg/L NAA and low concentrations (0.1 and 0.3 mg/L) of either BA, kinetin or iP. The highest average number of bulblets was produced on media with 0.1 mg/L NAA and 0.1 mg/L iP or BA. Medium supplemented with only 0.1 mg/L NAA gave the best results for rooting, while all concentrations of BA and high concentrations of iP and kinetin inhibited root formation. The results of BA inhibiting root formation are in accordance with data from TAKAYAMA *et al.* (1991) and BAIRU *et al.* (2007).

The induction of roots and bulblets in *Hyacinthus orientalis* grown in culture was investigated by SANIEWSKI *et al.* (1974). Bulblets grown on medium with either no growth regulators or containing 1 or 10 mg/L BA did not show any growth or bulblet formation. Medium supplemented with NAA resulted in the formation of callus which then developed into roots. Equal concentrations of BA and NAA inhibited callus and root formation but new bulb scales developed. New bulblet formation was greatest when medium was supplemented with 10 mg/L BA and 1 mg/L NAA.

MAESATO *et al.* (1994) studied the effect of plant growth regulators and culture environment on *in vitro* bulblet formation of *Lilium japonicum.* NAA (0.01 mg/L) and iP (0.01 mg/L) produced the best results, while zeatin showed the poorest response of the cytokinins tested. The highest number of bulblets were produced on media

supplemented with a combination of NAA and iP. The number of bulblets per scale explant was found to be related to auxin:cytokinin balance. Higher levels of cytokinins in combination with lower levels of auxin, produced the highest number of bulblets. However, the higher the growth regulator concentration, the higher the incidence of abnormal bulbs. iP formed larger and heavier bulbs in the dark, while media with NAA produced heavier bulbs in the light. MAESATO *et al.* (1994) therefore concluded that the supplementation of medium with growth regulators is necessary to achieve stimulatory responses and this growth regulator-induced response is finely tuned by the culture environment.

TAKAYAMA and MISAWA (1979) looked at the effect of various cultural conditions on *Lilium* bulb scales grown *in vitro*. Low concentrations (0.1 mg/L) of kinetin and NAA stimulated bulblet formation while higher concentrations (1 to 10 mg/L) inhibited the formation of bulblets. High concentrations of NAA stimulated root formation, however when kinetin was added, root formation was inhibited.

In vitro corm formation of *Gladiolus* was studied by DANTU and BHOJWANI (1995). With regards to shoot elongation, it was seen that the average length of the leaves was greatest when grown on media with 0.1 mg/L kinetin or 0.5 mg/L IAA. The highest number of roots per shoot developed on medium supplemented with 1 mg/L NAA, however, the roots were unbranched and the shoots developed some callus. IAA (0.25 and 0.5 mg/L) was the best auxin when it came to the number of roots per shoot and the length of roots.

In vitro bulb formation of *Narcissus asturiensis* was studied by SANTOS *et al.* (2002). Explants were cultured on MS medium supplemented with IBA and BA or NAA and BA. Both media induced shoots but shoot proliferation was the best on the NAA and BA medium. This data is in agreement with work done on other species of *Narcissus* by HUSSEY (1982b). Bulblets formed on both media, however, bulb induction and growth was higher on the medium supplemented with IBA. The rooting rate of bulblets was higher on IBA medium. YI *et al.* (2002) also found that medium with IBA

had the highest regeneration and growth rate of bulblets in *Hyacinthus orientalis* cv Carnegie compared to IAA. The greatest percent of root formation was observed with IBA rather than IAA. These results confirm those of SANTOS *et al.* (2002).

KROMER (1979) looked at the influence of exogenous growth regulators on *Canna indica* regeneration *in vitro*. When explants were placed on hormone free medium, no callus was formed and explants died after a few weeks. When auxins were included in the medium, callus formed. Well developed callus was observed on medium supplemented with 2,4-D, however, root formation was inhibited. A high percentage of explants with roots were observed on medium with IAA. NAA-containing media also produced explants with roots but not as many as medium containing IAA. The development of buds was stimulated when kinetin was included in the medium. The highest percentage of explants with buds developed on media supplemented with a combination of kinetin and IAA (KROMER, 1979).

The effect of auxins, cytokinins, gibberellins, abscisic acid and ethephon on regeneration and growth of bulblets on excised bulb scale segments of Hyacinth was studied by PIERIK and STEEGMANS (1975). With regards to the auxins, it was found that by increasing the concentration of IAA and IBA, the number of bulblets per explant increased. An increase in NAA, however, decreased the regeneration of bulblets but strongly promoted callus formation. High concentrations of IAA had no effect on bulblet growth while IBA reduced it. The cytokinins, kinetin and BA, hardly had an effect on bulblet regeneration and growth. Bulblet growth was reduced with an increase in GA_3 concentration. This was also found for GA_{4+7}, but inhibition of bulblet regeneration and growth started at a lower concentration. The results from ABA and ethephon showed that regeneration and bulblet growth were decreased proportionally to an increase in the concentration of these growth-regulating substances (PIERIK & STEEGMANS, 1975).

The initiation of adventitious bulbs in hyacinth depends on the appropriate balance of cytokinin and auxin within the bulb. It has been reported that IAA added to the culture

medium stimulates adventitious bulb formation from the bulb scale explants. An increase in IAA increases the number of bulbs produced as well as bulb weight (PIERIK & STEEGMANS, 1975; RUDNICKI, 1979). SANIEWSKI *et al.* (1974) found that a mixture of 1:10 auxin:cytokinin stimulates the differentiation of bulblets from adventitious bulbs as well as from florets. Auxin alone causes the formation of roots, while cytokinin (BA) alone did not affect the differentiation of either bulblets or roots. PIERIK and STEEGMANS (1975) found that the formation of bulbs and roots was strongly inhibited by gibberellins, ethylene and abscisic acid. Gibberellins were also found to inhibit bulblet formation in scooped out and scored hyacinth bulbs. Bulblets formed on GA-treated bulbs were abnormal and did not form leaves or roots (SANIEWSKI *et al.,* 1974; PIERIK & STEEGMANS, 1975).

Induction of dormancy and bulbing are part of the same mechanism and the mechanism is controlled by ABA. This has been observed in tissue-cultured lilies where the addition of ABA to the medium resulted in leaf formation being blocked and dormancy being induced. When an inhibitor of ABA-synthesis (fluridone) was added, bulb formation was blocked and the development of dormancy was prevented (DE KLERK *et al.,* 1992; BOONEKAMP, 1997). It has been reported that for bulbous crops, dormancy can be broken by a cold treatment or by supplementing the media with GA_3 or GA_{4+7} (BOONEKAMP, 1997). DANTU and BHOJWANI (1995) discovered that when ABA was included in the culture medium, corm formation in cultures of *Gladiolus* was inhibited.

KIM *et al.* (1994) looked at the effect abscisic acid has on dormancy development and bulb formation in lily plantlets. When ABA was added to the medium, plantlets consisted of only scales, no leaves formed. When the medium was supplemented with fluridone, swelling of the petioles was inhibited and the number of leaves per explant increased and the number of scales with no leaves decreased. KIM *et al.* (1994) also found that in lily, bulb formation and the occurrence of dormancy are closely linked.

SIMMONDS and WERRY (1987) tested the effect of GA_3 on bud formation of *Begonia* x *hiemalis*. The inclusion of GA_3 in the medium enhanced bud growth and petiole elongation was increased. It was therefore possible to harvest more buds from the GA_3 treatments. However, buds that developed on GA_3 treatments were difficult to root and of those that did root and were transferred to soil, only 50% developed into multistemmed plants. Due to the inhibitory affects of GA_3 on rooting and on adventitious bud production, SIMMONDS and WERRY (1987) concluded that GA_3 treatments were not suitable for micropropagation.

Jasmonic acid (JA) and its methyl ester (MeJA), called jasmonates, are plant growth regulators which show hormone like properties in different physiological processes connected with plant growth and development. The morphogenic processes that jasmonates are involved in, include germination and seedling development, flower development, tuberisation, bulb formation, determination of plant structure, tendril coiling, leaf senescence and fruit ripening (GROSS & PARTHIER, 1994; CREELMAN & MULLET, 1997; KODA, 1997; ANANIEVA *et al.,* 2004). Research showed that JA dramatically alters gene expression and in response to abiotic and biotic stresses such as wounding, pathogen attack and desiccation, JA/MeJA is accumulated (GASPAR *et al.,* 1996). This implies that jasmonates play a role in a plants defense system or as signals involved in stress-related responses. Therefore, jasmonates have a dual role; they play a part in the development of the plant as well as in the plant's defense system (CREELMAN & MULLET, 1997; ANANIEVA *et al.,* 2004).

Jasmonic acid and methyl-jasmonate are also known to have various effects on the growth of cells. JA and MeJA inhibit cell division and cell elongation, but promote cell expansion. It is believed that jasmonates regulate cell division and the direction of cell expansion, and thus lead to defined shapes of organs or tissues (KODA, 1997). When tissue discs from potato tubers were placed on medium supplemented with JA, they began to swell. After five days in culture, the fresh weight of the discs had doubled. On examination under a light microscope, it was seen that the swelling was due to the expansion and not the division of cells. KODA (1997) claims that the

expansion-inducing activity appears to be specific to jasmonates as different plant growth regulators such as IAA, GA_3, ABA, BA and ethylene, had no effect on the size of cells. Therefore, the use of jasmonates could be beneficial for the tissue culture of bulbous and tuberous plants.

DEBELJAK *et al.* (2002) studied the induction of tuberisation *in vitro* with jasmonic acid and sucrose on an Australian terrestrial orchid, *Pterostylis sanguinea*. From the results obtained, it was observed that a significantly higher proportion of seedlings produced tubers when the medium was supplemented with 5 g/L sucrose and 1 mg/L JA. When 1 mg/L JA was added to the medium, 86% of the plants produced tubers compared to 28.5% in the control. From these results, DEBELJAK *et al.* (2002) showed that tuberisation in terrestrial orchids can be enhanced with the addition of JA in the culture medium.

The influence of jasmonic acid on shoot and bulb formation in tissue culture of garlic (*Allium sativum* cv Ptuj) was studied by RAVNIKAR *et al.* (1993). When the medium contained both JA and iP, a very high number of shoots developed. The shoots were transferred onto medium with only JA and a large number of bulbs formed. The increase in bulbs corresponded with the increase in JA concentration in the medium. Although the average number of shoots and bulbs was highest on medium supplemented with 2 mg/L JA, it is suggested that a concentration of 1 mg/L or 1.5 mg/L JA be used as the bulbs were largest at these concentrations. The results show that the introduction of JA in the micropropagation of garlic has beneficial effects on shoot and bulb formation (RAVNIKAR *et al.,* 1993).

SANTOS and SALEMA (2000) looked at the promotion of bulb formation in shoot cultures of *Narcissus triandrus* by jasmonic acid. Media supplemented with JA and iP or JA and NAA produced a high multiplication of leaves, however, leaf formation was low on medium containing just JA. The highest number of bulbs and largest bulbs, with the largest diameters, formed on medium with JA alone compared to media with JA and iP or JA and NAA. Medium supplemented with only NAA produced a few

small bulbs but the bulbs were elongated in shape. The data obtained from these experiments show that JA promotes *in vitro* bulb formation in shoot cultures of *N. triandrus* and it suggests that JA might play a significant role in the formation and enlargement of bulbs in *Narcissus* plants.

Plant growth regulators including growth retardants play an important role in plant propagation especially in tissue culture. Growth retardants are involved in various developmental processes. These include elongation of leaves, stems and roots or callus formation. Inhibitors of gibberellic acid biosynthesis such as ancymidol, flurprimidol and paclobutrazol (PAC) are used in tissue culture especially with bulbous plants. Growth retardants are useful in preventing leaf growth and shoot vitrification in liquid culture as well as reducing the time taken to produce storage organs *in vitro* (ZIV, 1989; ILAN *et al.,* 1995; ZIV, 1997; ILCZUK *et al.,* 2005). ILCZUK *et al.* (2005) tested the effect of flurprimidol on the propagation of *Hippeastrum* x *chmielii* Chm. When the medium was supplemented with the growth retardant, flurprimidol, the propagation rate was significantly increased and the number of bulblets obtained was nearly double compared to explants grown on media without flurprimidol. The size of the newly developed bulblets was also affected by flurprimidol. Bulblets were much bigger in size. Root growth was also promoted when flurprimidol was present in the medium. Similar results have been reported for *Narcissus, Nerine, Allium* and *Gladiolus* with other growth retardants (ZIV, 1989; ILCZUK *et al.,* 2005).

Enhanced shoot and cormlet proliferation in liquid cultured *Gladiolus* buds by growth retardants was looked at by ZIV (1989). Bud explants grown in liquid media supplemented with the growth retardants daminozide, ancymidol or paclobutrazol (PAC), all developed into large bud aggregates without leaves. PAC induced the highest number of buds per explant. The buds developed into protocorms and after transferring to solid MS media, cormlets formed. PAC however had a carry over effect and inhibited leaf elongation even after transplanting onto a medium without the growth retardant.

LILIEN-KIPNIS and ZIV (1992) studied the proliferation and regeneration of *Nerine*. The regeneration rate of proliferating callus clusters was low and it was thought that PAC was having an inhibitory effect on the development of plantlets. A decrease in the growth value of proliferating clusters was also observed. Therefore, PAC was removed from the medium. The growth rate significantly increased when clusters were grown on medium without PAC. LILIEN-KIPNIS *et al.* (1994) also found a change in the morphology of the proliferating tissue of *Nerine* when PAC was omitted from the medium. PAC-free medium induced proembryogenic clusters.

These studies described above illustrate the variability of the effects of different plant growth regulators on *in vitro* cultures. Importantly, the interactions between different classes of plant growth regulators, and with different environmental conditions, have an affect on culture growth and development. The impact of endogenous levels of hormones on the impact of exogenous application should not be excluded as different explants at different times may contain different endogenous levels of hormones which will also have an impact on the response *in vitro*.

5.2 MATERIALS AND METHODS

Leaf explants from previously established *in vitro* plantlets were inoculated onto 40 ml MURASHIGE and SKOOG (1962) basal medium containing various plant growth regulators at various concentrations, namely 1, 2 and 5 mg/L (Table 5.1). The medium was supplemented with 100 mg/L myo-inositol, 30 g/L sucrose and solidified with 8 g/L agar. The pH of the medium was adjusted to 5.8 with diluted KOH or NaOH before autoclaving at 121 ℃ and 103 kPa for 20 min. The culture bottles (6 cm diameter, 10 cm high) were stored for 3 months in environmental chambers (Convirons) at 20 ℃ with a 16 h photoperiod. The Conorivons contained Osram L58W/640 cool white fluorescent bulbs and the light intensity range in the growth chambers was 70 to 90 µmol/m^2/s. After 3 months the bulblets were removed, and bulblet mass and diameter were determined.

Table 5.1: Plant growth regulators tested at various concentrations.

Auxins	Cytokinins	Other PGRs
NAA	BA	GA_3
IAA	iP	GA_{4+7}
IBA	Zeatin	ABA
2,4-D	*m*T	MeJa
phenylacetic acid (PAA)		PAC

Five explants were placed in each jar with 8 jars per treatment. Data collected were subjected to one-way analysis of variance (ANOVA). Where there is a significant difference (p=0.05), the means were separated using Duncan Multiple Range Test (DMRT). Data analysis was done using SPSS version 15.0.

5.3 RESULTS

Initiation of bulblets occurred directly on the leaf explants in the form of small protrusions which after a month developed into small bulblets. Medium supplemented with 1 mg/L 2,4-D initiated the most shoots and bulblets per explant (an average of 1.53). The explants grown on medium with 2,4-D swelled and became a yellowish brown colour. Although the number of bulbs was highest on 2,4-D-containing medium, they were small in diameter (1 to 3 mm) compared to 1 mg/L IBA. However, when 2,4-D was compared with the control (2.28 mm) the sizes of the bulbs were not much bigger (Table 5.2).

The best bulbs, in terms of diameter and weight, were produced on both 1 mg/L NAA and 1 mg/L IBA. These bulbs were bright green in colour, large in size, had good leaves and the roots were long and well established. Fewer bulbs were produced per explant on these treatments, however, the bulblets formed were larger than those formed on the control and 2,4-D. Bulblets produced on medium supplemented with 1 mg/L IBA had an average bulb diameter of 4.36 mm and an average weight of 79.07 mg, while bulblets produced on 1 mg/L NAA had an average diameter of 3.51 and average bulb weight of 51.99 mg (Table 5.2).

Of the cytokinins tested 5 mg/L BA produced the highest number of bulblets per leaf explants followed by 2 mg/L zeatin. Bulblets grown on medium supplemented with 5 mg/L BA had the largest diameter, 2.10 mm, and heaviest weight, 15.85 mg, (Figure 5.1 and Figure 5.2). The cytokinin to produce the lowest results, compared with the other cytokinins tested, in terms of bulb number, diameter and weight was 1 mg/L iP.

The plant growth regulators GA_{4+7} and MeJA produced poor results for number of bulblets induced per explant (Figure 5.1). The explants generally turned brown in colour and showed no response. GA_{4+7} and MeJA never produced results that were better than the control with regards to mean number of bulbs per explant, mean bulb diameter and mean bulb weight. The control produced an average of 0.35 bulbs per leaf explant with an average bulb diameter of 2.28 mm and average weight of 16.94 mg. No bulblets were produced on media supplemented with 5 mg/L GA_{4+7}.

To confirm the optimum treatment, the product value was calculated. The product value is calculated by converting the percent bulb induction to a proportion and then multipling by the bulb mass. This enables a more consistent ranking of treatments (ASCOUGH, 2008). From Table 5.3 it can be seen that 1 mg/L IBA was the optimum hormone treatment as it had the highest product value, 67.21.

Table 5.2: The effect of various plant growth regulators (PGR) and concentrations on the average number of bulblets produced per explant and the mean size and weight of the bulblets. Significant differences are shown.

PGR (mg/L)	Mean no. of bulbs/explants	Mean bulb diameter (mm)	Mean bulb weight (mg)
Control	0.35 ± 0.063^{ghijklm}	2.28 ± 0.094^{cdefghij}	16.94 ± 1.85^{defghi}
1 NAA	1.13 ± 0.164^{bc}	3.51 ± 0.316^{ab}	51.99 ± 11.00^{b}
2 NAA	0.78 ± 0.175^{cdef}	2.68 ± 0.193^{bcdefg}	29.12 ± 5.26^{cde}
5 NAA	0.43 ± 0.080^{fghijklm}	2.41 ± 0.164^{bcdefghi}	18.68 ± 4.98^{cdefghi}
1 2,4-D	1.53 ± 0.501^{a}	2.76 ± 0.124^{bcdef}	27.77 ± 2.79^{cdef}
2 2,4-D	0.63 ± 0.139^{efghi}	2.36 ± 0.117^{cdefghij}	21.15 ± 2.26^{cdefgh}
5 2,4-D	0.25 ± 0.091^{hijklm}	1.54 ± 0.467^{ghij}	9.63 ± 3.38^{efghi}

Table 5.2: Continued

PGR (mg/L)	Mean no. of bulbs/explants	Mean bulb diameter (mm)	Mean bulb weight (mg)
1 BA	0.45±0.098^{fghijkl}	1.84±0.158^{defghij}	12.94±1.81^{efghi}
2 BA	0.38±0.110^{fghijklm}	1.76±0.409^{efghij}	14.53±3.88^{defghi}
5 BA	0.73±0.131^{defg}	2.10±0.339^{cdefghij}	15.85±3.86^{defghi}
1 IAA	0.38±0.116^{fghijklm}	1.83±0.575^{defghij}	22.34±9.36^{cdefg}
2 IAA	0.65±0.091^{efgh}	2.95±0.258^{bcd}	37.99±8.07^{bc}
5 IAA	0.60±0.125^{efghij}	2.52±0.484^{bcdefgh}	30.28±8.41^{cde}
1 IBA	1.08±0.106^{bcd}	4.36±0.390^{a}	79.07±17.35^{a}
2 IBA	0.93±0.239^{bcde}	2.84±0.455^{bcde}	34.14±8.23^{bcd}
5 IBA	1.28±0.160^{ab}	3.51±0.190^{ab}	50.14±8.03^{b}
1 GA_3	0.40±0.120^{fghijklm}	2.81±0.684^{bcde}	38.35±14.17^{bc}
2 GA_3	0.28±0.037^{hijklm}	3.03±0.334^{bc}	23.81±7.62^{cdefg}
5 GA_3	0.18±0.059^{jklm}	1.74±0.566^{efghij}	10.38±5.62^{efghi}
1 GA_{4+7}	0.23±0.070^{hijklm}	1.51±0.361^{ghij}	6.73±1.86^{ghi}
2 GA_{4+7}	0.05±0.033^{lm}	0.39±0.258^{kl}	1.38±0.94^{hi}
5 GA_{4+7}	0.00±0.000^{m}	0.00±0.000^{l}	0.00±0.00^{i}
1 PAA	0.25±0.082^{hijklm}	1.28±0.416^{ijk}	12.04±4.92^{efghi}
2 PAA	0.28±0.065^{hijklm}	2.08±0.308^{cdefghij}	20.35±3.61^{cdefghi}
5 PAA	0.20±0.085^{ijklm}	1.15±0.446^{jk}	10.25±4.31^{efghi}
1 Zeatin	0.55±0.050^{efghijk}	1.82±0.106^{defghij}	14.53±1.60^{defghi}
2 Zeatin	0.63±0.059^{efghi}	1.59±0.062^{fghij}	10.02±0.90^{efghi}
5 Zeatin	0.40±0.085^{fghijklm}	1.52±0.235^{ghij}	11.21±1.80^{efghi}
1 iP	0.20±0.065^{ijklm}	1.18±0.353^{jk}	7.94±2.47^{fghi}
2 iP	0.33±0.075^{ghijklm}	1.95±0.072^{cdefghij}	14.47±0.76^{defghi}
5 iP	0.53±0.075^{efghijk}	1.92±0.068^{cdefghij}	13.02±1.54^{efghi}
1 ABA	0.33±0.075^{ghijklm}	1.40±0.218^{hijk}	8.35±1.78^{fghi}
2 ABA	0.20±0.053^{ijklm}	1.22±0.291^{ijk}	7.13±1.92^{fghi}
5 ABA	0.30±0.065^{ghijklm}	1.22±0.274^{ijk}	5.88±1.45^{ghi}
1 *m*T	0.35±0.033^{ghijklm}	1.66±0.075^{efghij}	10.00±1.33^{efghi}
2 *m*T	0.55±0.118^{efghijk}	1.46±0.222^{hijk}	8.39±1.59^{fghi}
5 *m*T	0.53±0.106^{efghijk}	1.60±0.243^{fghij}	11.41±2.17^{efghi}
1 MeJa	0.23±0.070^{hijklm}	1.81±0.574^{defghij}	17.56±8.37^{defghi}
2 MeJa	0.20±0.065^{ijklm}	1.65±0.492^{efghij}	10.56±3.53^{efghi}
5 MeJa	0.15±0.033^{klm}	1.62±0.368^{fghij}	10.38±2.78^{efghi}
1 PAC	0.50±0.038^{fghijk}	2.26±0.134^{cdefghij}	19.79±2.59^{cdefghi}
2 PAC	0.28±0.053^{hijklm}	2.02±0.333^{cdefghij}	21.13±5.06^{cdefgh}
5 PAC	0.18±0.059^{jklm}	1.61±0.555^{fghij}	18.75±8.26^{cdefghi}

Mean values in the same column followed by the same letter(s) are not significantly different (p=0.05).

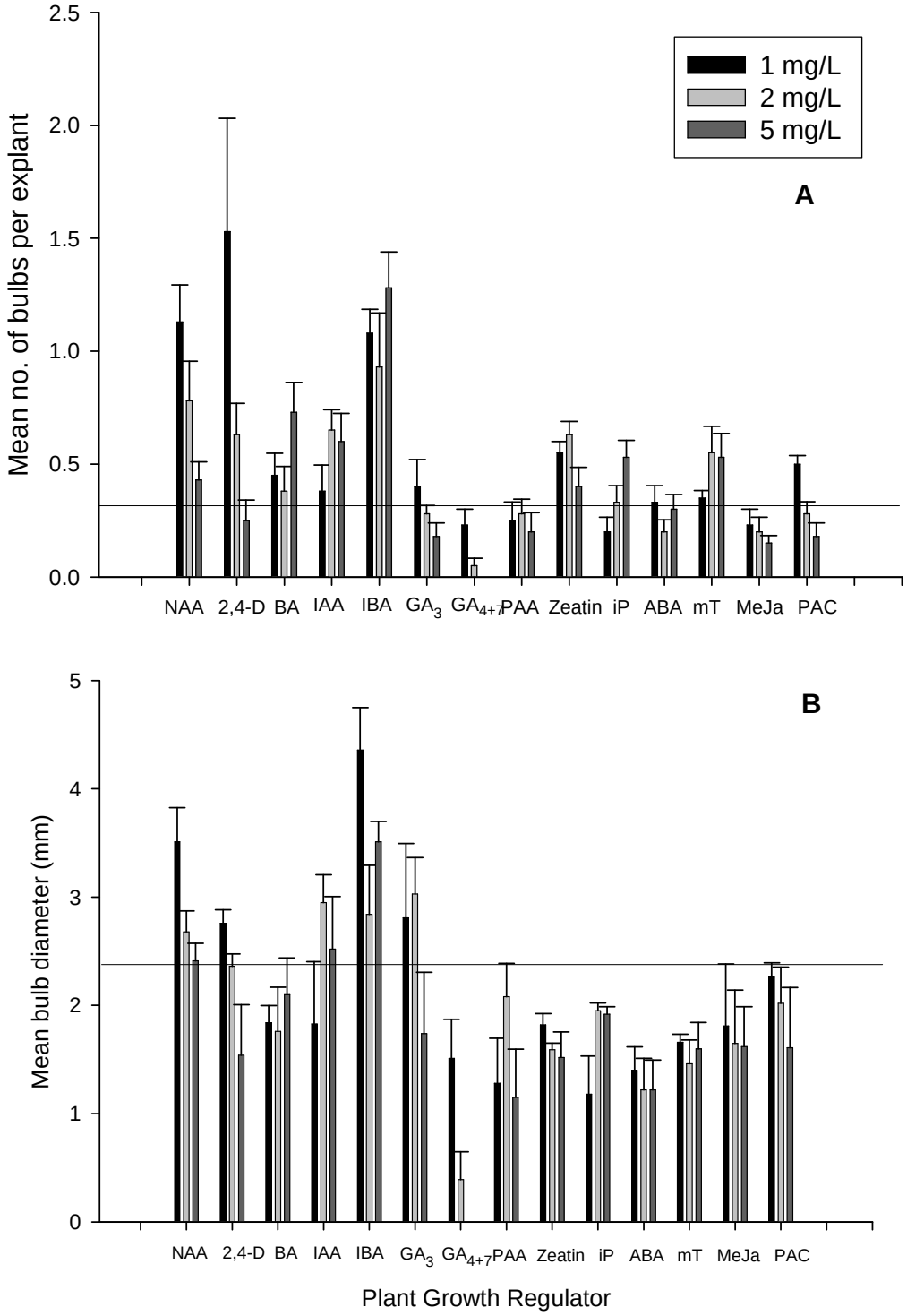


Figure 5.1: The mean number of bulblets per explant (A) and mean bulb diameter (B) for the various hormone treatments using *E. zambesiaca* leaf explants.

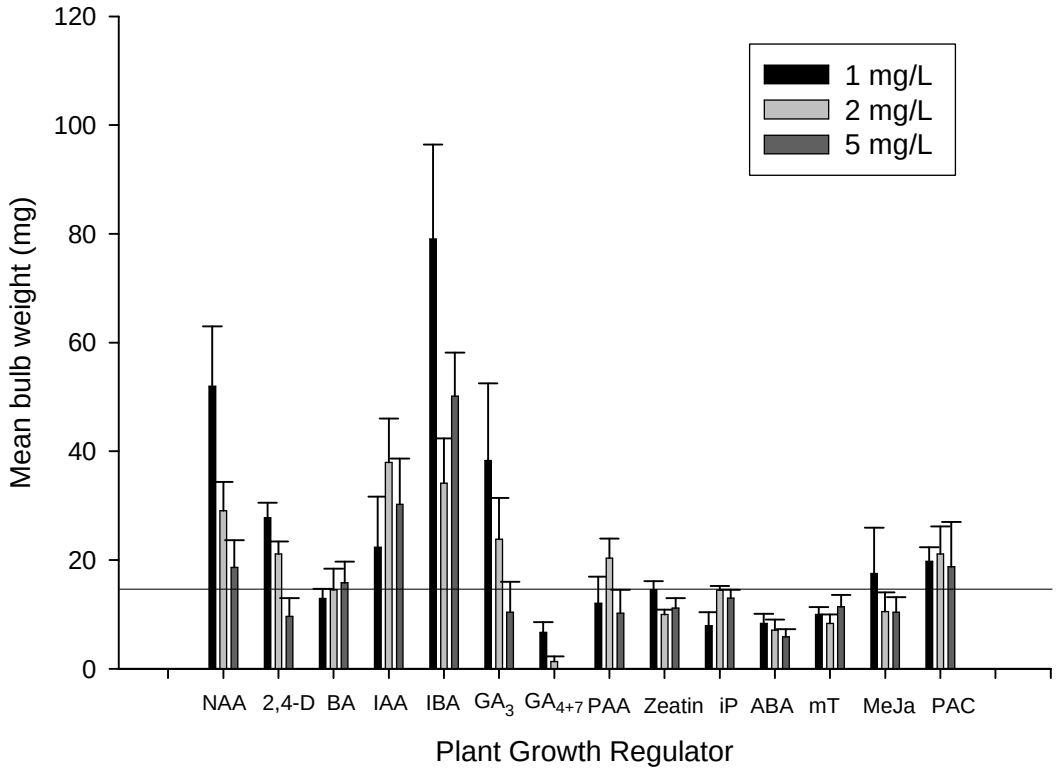


Figure 5.2: Effect of various hormones on mean bulblet weight of bulblets induced on *E. zambesica* leaf explants.

Table 5.3: Product value showing the optimum hormone treatments for bulblet induction on *E. zambesiaca* leaf explants.

Hormone	Concentration (mg/L)	Product value
Control	0	4.66
NAA	1	40.29
	2	15.29
	5	6.07
2,4-D	1	17.36
	2	8.99
	5	2.41
BA	1	5.18
	2	3.27
	5	7.93
IAA	1	7.82
	2	20.89
	5	13.63

Table 5.3: Continued

Hormone	Concentration (mg/L)	Product value
IBA	1	67.21
	2	17.07
	5	38.86
GA_3	1	13.42
	2	6.55
	5	1.82
GA_{4+7}	1	1.51
	2	0.07
	5	0.00
PAA	1	3.01
	2	5.60
	5	2.05
Zeatin	1	7.63
	2	5.76
	5	4.20
iP	1	1.59
	2	4.34
	5	6.51
ABA	1	2.72
	2	1.43
	5	1.76
*m*T	1	3.50
	2	3.77
	5	5.42
MeJa	1	3.95
	2	2.11
	5	1.56
PAC	1	9.90
	2	4.75
	5	3.28

5.4 DISCUSSION

From the above data it can be seen that the most successful hormone treatment was 1 mg/L IBA, which had the highest product value of 67.21 (Table 5.3). Treatments of 1 mg/L NAA, 5 mg/L IBA and 2 mg/L IAA also produced good results, with product values of 40.29, 38.86 and 20.89, respectively (Table 5.3). Of all the plant growth regulators tested, three of the five auxins used gave the best results. It has been reported that the addition of auxins, even at low concentrations, significantly increase the number of bulblets obtained *in vitro*.

IBA is a very stable plant growth regulator and it is slowly metabolized, however, it is poorly transported around the plant. Due to poor transportation IBA possibly stays at the site of contact i.e. where the leaf explant touches the culture medium, and is metabolized slowly and does not interfere with endogenous auxins. Therefore, better bulblet induction results were obtained with IBA-containing medium compared with other plant growth regulators.

DABROWSKI *et al.* (1992) found similar results with regards to auxins producing good results, however, they reported that the addition of both auxins and cytokinins to the medium increased the regeneration ability of explants more. In these experiments on *E. zambesiaca* combinations of auxins and cytokinins were not tested.

In vitro bulb formation of *Narcissus asturiensis* was studied by SANTOS *et al.* (2002) and it was found that bulb induction and growth was higher on the medium supplemented with IBA. The rooting rate of bulblets was higher on IBA containing medium. YI *et al.* (2002) also found that medium with IBA had the highest regeneration and growth rate of bulblets in *Hyacinthus orientalis* cv. Carnegie compared to IAA. The greatest percentage of root formation was observed with IBA rather than IAA. These results are in agreement with our data, where IBA was the hormone that produced the best results for the induction of bulblets.

It has also been reported that IAA added to the culture medium stimulates adventitious bulb formation from the bulb scale explants of hyacinth. An increase in IAA increases the number of bulbs produced as well as bulb weight (PIERIK & STEEGMANS, 1975; RUDNICKI, 1979). This is in accordance with the data recorded for *E. zambesiaca*. Both 2 and 5 mg/L IAA produced better results than 1 mg/L. Not only were more bulbs produced per explant, but the size of the bulblets were larger at the higher concentrations.

BAIRU *et al.* (2007) looked at optimizing the micropropagation protocol for *Aloe polyphylla* by using *meta*-topolin instead of BA and found that at low concentrations, BA produced more shoots however, as the concentration increased, larger numbers of shoots were obtained from the *m*T treatments. This is not true for the experiments on *E. zambesiaca*. BA generally produced more bulbs compared with the number of bulbs produced from *m*T treatments. Of the BA treatments, a concentration of 5 mg/L BA produced better results than 1 and 2 mg/L. At 5 mg/L BA, the average number of bulblets per explant was 0.73 and the bulblets had a mean diameter of 2.1 mm and mean weight of 15.85 mg. Both TAKAYAMA *et al.* (1991) and ULRICH *et al.* (1999) found that high concentrations of BA stimulated the formation of bulbs. ULRICH *et al.* (1999) reported that the greatest shoot formation of *Crinum* 'Ellen Bosanquet' was on medium with 5 mg/L BA. This is in agreement with the results obtained from this work on *E. zambesiaca* where the highest BA concentration produced the largest number of bulbs per explant compared to the other BA treatments.

All NAA treatments produced better results, with regards to average number of bulbs per leaf explant, mean bulb diameter and mean bulb weight, compared with the results obtained from the control. TAKAYAMA *et al.* (1991) found bulb regeneration of *Hyacinthus orientalis* was stimulated by a combination of 10 mg/L BA and 0.1 mg/L NAA. Low concentrations of BA and either no NAA or high concentrations of NAA did not stimulate bulblet formation. A high concentration of NAA enhanced the development of regenerative callus. TAKAYAMA and MISAWA (1979) found the

results by TAKAYAMA *et al.* (1991) to be true for the bulblet induction of *Lilium*. All concentrations of NAA induced bulblets on *E. zambesiaca* leaf explants, however, as the concentration increased from 1 mg/L to 5 mg/L bulblet regeneration and growth decreased from 1.13 to 0.43 bulbs per explant and 51.99 to 18.68 mg respectively. In *E. zambesiaca* callus formation was not promoted by a high (5 mg/L) NAA concentration.

The gibberellin (GA_{4+7}) produced some of the poorest results, while 1 mg/L GA_3 produced fairly good results. Although few bulblets formed on medium with GA_3, the bulblets that did form were a fair size (1 to 3 mm) and had long thin leaves. The GA_{4+7} treatments produced some of the poorest results. At a concentration of 5 mg/L GA_{4+7}, no bulblets were produced. PIERIK and STEEGMANS (1975) reported that bulblet growth was reduced with an increase in GA_3 concentration and this was also found with GA_{4+7} but inhibition of bulblet regeneration and growth started at a lower concentration. This is also true for *E. zambesiaca* as increasing concentration decreased bulblet regeneration and growth.

The results from experiments on hyacinth showed that regeneration and bulblet growth are decreased proportionally to an increase in the concentration of ABA (PIERIK & STEEGMANS, 1975). From Table 5.2 it can be seen that what PIERIK and STEEGMANS (1975) reported is in accordance with the results recorded for the effect of ABA on *E. zambesiaca*. As the concentration increased from 1 to 5 mg/L ABA, the size of the bulblets decreased.

One of the morphogenic processes that jasmonates (JA or MeJA) are involved in is bulb formation. When leaf explants of *E. zambesiaca* were cultured on media containing MeJA, very few bulblets formed. Bulblets had an average diameter of 1.69 mm and an average weight of 12.8 mg. When shoots of *Allium sativum* cv Ptuj were transferred onto medium with JA a large number of bulbs formed and it was observed that an increase in bulbs corresponded with the increase in JA concentration in the medium (RAVNIKAR *et al.,* 1993). SANTOS and SALEMA (2000) also found JA to

have a stimulatory effect on bulb formation in shoot cultures of *Narcissus triandrus*. The largest number of bulbs and biggest bulbs, with the greatest diameters, formed on medium with JA alone compared to media with JA and iP or JA and NAA. The results from both RAVNIKAR *et al.* (1993) and SANTOS and SALEMA (2000) contradicts the results obtained in the present experiments on *E. zambesiaca*.

Growth retardants, such as PAC, are often used in tissue culture as they are useful in preventing leaf growth and shoot vitrification. Within the PAC treatments, 1 mg/L PAC was better than 2 and 5 mg/L in terms of mean number of bulbs per explant and mean bulb diameter. The heaviest bulbs were formed on medium supplemented with 2 mg/L PAC. However, when PAC treatments are compared to the control, only 1 mg/L PAC produced more bulbs per explant but all PAC treatments produced heavier bulbs than those grown on the control medium. All the bulblets that developed on medium containing PAC had fewer leaves and the leaves were short in length (they appeared to be stunted). LILIEN-KIPNIS and ZIV (1992) found PAC had an inhibitory effect on the initiation and development of *Nerine* plantlets compared to PAC free medium. This is in contradiction to our findings as bulblet formation was obtained with the presence of PAC in the medium culture. Medium containing 1 mg/L PAC produced better results than the control.

The same composition of growth regulators in the culture medium may cause different reactions in different species and even cultivars, or similar reactions may be seen when sections of different organs are placed on the same medium. These variations are due to the fact that different species and sections of various organs, differ in their ability to produce endogenous growth substances or they differ in their content at the time of isolation (HEMPEL, 1979). Variations could also be a result of the species or section of organ's ability to recognize and take up exogenous hormones as well as their ability to transport, metabolize and respond to the exogenous hormones. Therefore, the use of plant growth regulators in tissue culture is an art. It is not possible to dictate a particular concentration or plant growth regulator to be used in any single case.

5.5 SUMMARY

- 1 mg/L 2,4-D produced the greatest number of bulblets per explant
- 1 mg/L IBA produced the bulbs with the widest diameter
- 1 mg/L IBA produced the heaviest bulbs
- 1 mg/L IBA is the optimum hormone treatment for the induction of bulblets in *Eucomis zambesiaca*

Chapter 6
LIQUID CULTURE

6.1 INTRODUCTION

Micropropagation was originally used only for the large scale production of ornamental plants, however, today the technique is being used to propagate crops, trees as well as medicinal and aromatic plants. The micropropagation technique is not only used to produce plants which are difficult to propagate through conventional methods but also for conservation purposes. Important, rare plant species that are threatened or nearly extinct, are being multiplied *in vitro* using this biotechnological tool (MEHROTRA *et al.,* 2007).

Although the micropropagation technique is very advantageous, a number of problems do exist. The micropropagation technique is expensive, time consuming and labour intensive. The challenge today is to come up with a way to reduce production costs and in so doing, improve the production efficiency. A number of cost reduction strategies have been developed. One of which is liquid culture medium. Liquid culture can be used alone or in combination with solid culture medium forming a bilayer (TAKAYAMA *et al.,* 1991; MEHROTRA *et al.,* 2007).

Liquid culture has been less used for micropropagation even though it has certain merits. Firstly, it is more cost effective as a gelling agent is not required. Secondly, growth and multiplication rates of shoots, roots, bulblets and somatic embryos are enhanced as in liquid culture the explant is covered by the medium which stimulates and facilitates the uptake of nutrients and plant growth regulators. Thirdly, agitation of the medium allows for, the even distribution of nutrients and growth regulators which prevent depletion zones around the explant, as well as for better aeration. Improved growth rate in liquid cultures is also believed to be as a result of greater water availability, a more gradual pH change during culture and the removal of polarity. The

last advantage is the reduction in the deleterious effect of toxins. When substances are released into the medium in solidified cultures they remain in close proximity of the explant, whereas in liquid culture the toxins are quickly diluted and so decrease the potential inhibitory effect (ASCOUGH & FENNELL, 2004; MEHROTRA *et al.,* 2007).

Liquid culture does have a few disadvantages. The most common being vitrification, otherwise known as hyperhydricity. Vitrification is a result of the explant being entirely covered by the medium and is therefore oxygen deficient. This leads to the development of abnormal leaves, such as elongated leaves that are hyperhydric. Vitrification can be avoided by either making use of the surface tension and floating explants, so that developing tissues do not get submerged in the medium or by bubbling sterile air through the liquid medium (ZIV, 1991; DEBERGH *et al.,* 1992; ASCOUGH & FENNELL, 2004; MEHROTRA *et al.,* 2007). Other problems include aeration and shearing forces which damage cells and break up cell aggregates. Aeration is a problem as oxygen levels are low and so carbon dioxide quickly builds up and may dissolve in the medium which results in a decrease in the pH (ASCOUGH & FENNELL, 2004).

There are three main liquid culture systems that are used by researchers today. They are liquid shake culture, temporary immersion and bioreactors. Liquid shake cultures are when explants are placed in liquid medium and the culture flask is then shaken or rotated. This method is better than static liquid culture as shaking the culture flask improves aeration. Temporary immersion is as the name implies, when explants are temporarily immersed in liquid culture or it can be defined as the flooding of plant tissue at regular intervals. Optimum immersion cycles can be obtained by adjusting both the duration and frequency of the immersions. Temporary immersions are beneficial for plant growth as aeration is good, it allows for the removal of deleterious gases like CO_2 and ethylene and the problem of shearing is eliminated as the cultures are not shaken (ILCZUK *et al.,* 2005). “Bioreactors are self contained, sterile environments which capitalize on liquid nutrient or liquid/air inflow and out flow

systems, designed for intensive culture and affording maximal opportunity for monitoring and control over micro environmental conditions" (MEHROTRA *et al.,* 2007). According to ASCOUGH and FENNELL (2004) there are three instances when bioreactors are used. They are: suspension cultures for regenerating somatic embryos, suspension cultures used for production of secondary metabolites and for the production of plantlets. The advantages of bioreactors include the ease of scaling up in less time, the ability to program the system to exchange media, supply bubbles and stir the culture thus reducing labour costs and the elimination of physiological disorders of shoot and leaf hyperhydricity.

LILIEN-KIPNIS and ZIV (1992) studied the proliferation and regeneration of *Nerine* in liquid culture. It was found that the growth value of meristematic clusters was four to six times higher in bioreactors than that obtained from shaken flasks. ILCZUK *et al.* (2005) on the other hand found that the temporary immersion system gave the best results. *In vitro* propagation of *Hippeastrum* x *chmielii* Chm. in solid or liquid medium and in temporary immersion systems was examined by ILCZUK *et al.* (2005). On solid medium 3.9 new bulblets were formed per initial bulb while liquid culture and temporary immersion systems produced 6.3-6.5 bulblets. The bulblets produced in liquid culture had higher fresh masses compared to those produced on solid medium. As no malformations of the bulblets were observed and further development was fast, ILCZUK *et al.* (2005) recommend the use of either liquid culture or temporay immersion systems for the propagation of *Hippeastrum* x *chmielii* Chm.

TAKAYAMA *et al.* (1991), ILAN *et al.* (1995) and NHUT *et al.* (2004) all found liquid shake culture gave the best results with regards to propagation of shoots and bulbs. TAKAYAMA *et al.* (1991) worked on *Hyacinthus orientalis* to improve bulblet regeneration and their growth *in vitro*. Bulb weight increased 10-fold in liquid shake culture compared to those grown on solid media. However, root growth and growth of regenerative callus was greater on solid media. ILAN *et al.,* (1995) looked at the propagation and corm development of several species of *Brodiaea* in liquid cultures. The production and growth of protocorm-like bodies were greater when they were

agitated and circulated freely in bioreactors compared with the protocorm-like bodies that were static and not shaken. It is believed that circulation and agitation led to oxygen enrichment of the medium which affected and improved growth as well as prevented polar orientation in growing plant tissues (ILAN *et al.,* 1995). The micropropagation of *Gladiolus* by liquid shake culture was researched by NHUT *et al.* (2004). In liquid shake culture, all the shoots produced new shoots and a continuous mass shoot propagation system was established. Shoot clusters were harvested and divided into single shoots and then re-cultured. This cycle was repeated three times and an average of 216 shoots were produced. Therefore, NHUT *et al.* (2004) reported that liquid shake culture can produce a large number of uniform shoots and in so doing, reduce the cost of micropropagated plants.

The results obtained by VARSHNEY *et al.* (2000) contradicted that of TAKAYAMA *et al.* (1991), ILAN *et al.* (1995) and NHUT *et al.* (2004), who found liquid shake culture to be the better system. VARSHNEY *et al.* (2000) developed a protocol for the *in vitro* mass propagation of two cultivars of Asiatic lily hybrids and compared liquid stationary cultures to liquid shake cultures. In the shake cultures, the bulb scale clusters turned brown. The scales were also damaged by shearing and possible leaching into the medium. In contrast, stationary cultures resulted in better scale proliferation with no leaching. Thus, VARSHNEY *et al.* (2000) concluded that for scale proliferation, liquid stationary culture is more effective than the shake culture.

The shake liquid culture as an alternative way to the multiplication of *Narcissus* plants was investigated by BERGOÑÓN *et al.* (1992). Shoot clusters that were cultured in liquid media produced a better proliferation of shoots compared to those cultured on solid media. The shoots in liquid media were also larger in size, however, the root system was better developed in shoots cultured on the solid medium. Vitrification problems and other morphogenic abnormalities of the shoots were not observed in liquid culture which allows the liquid shake culture to be considered as a successful propagation method for *Narcissus* plants. BERGOÑÓN *et al.* (1996) also carried out experiments on *Narcissus confuses* in liquid shake medium. It was found that liquid

medium stimulated the development of bulblets from shoot clumps in the same way as observed in other bulbous plants (BERGOÑÓN *et al.,* 1992).

Micropropagation of the rare *Eucrosia stricklandii* by twin-scaling and shake liquid culture was studied by COLQUE *et al.* (2002). Liquid medium resulted in faster growth of the shoot-clumps in comparison with the bulblets grown in semi-solid medium. Good bulbling and leaf growth was obtained from liquid media supplemented with 0.263 M sucrose. The biomass was also increased by culturing the explants in liquid medium which was previously reported for *Narcissus*. (BERGOÑÓN *et al.,* 1996; SELLÉS *et al.,* 1997). This research proved that shake liquid culture was an efficient system for multiplying *E. stricklandii*.

MORÁN *et al.* (2003) looked at the mass propagation of *Cyrtanthus clavatus* and *Cyrtanthus spiralis* using liquid culture. After shoot multiplication the bulbing process was carried out on both solid and liquid media. Large bulblets were placed on solid media while the smallest bulbs were cultured in liquid media to promote growth. Bulblets of both *C. clavatus* and *C. spiralis* grown in liquid media that contained 30 and 60 g/L of sucrose had the highest growth index and long, green leaves were produced. The bulblets in liquid culture formed mainly bulbs, however, buds also formed. The liquid medium increased the biomass of the bulbs in a higher proportion and in a shorter time period compared to those grown on solid media. MORÁN *et al.* (2003) determined that liquid culture with 30 or 60 g/L sucrose can be used to accelerate biomass gain and the bulbing of small sized shoots for both *C. clavatus* and *C. spiralis.*

SIMMONDS and WERRY (1987) used liquid shake culture to improve micropropagation of *Begonia* x *hiemalis*. After 9 weeks of petiole explants being grown on solid medium, a small number of buds had formed and only a few were large enough to be used for rooting. SIMMONDS and WERRY (1987) found that if the explants were placed in liquid shake culture after being on agar for 3 or 6 weeks, bud growth was enhanced which led to a larger number of shoots available for

rooting. The improved production of larger shoots allows for easy handling on a commercial scale.

HAN *et al.* (2004) studied the effect of the addition of liquid medium on the *in vitro* shoot formation of *Lilium longiflorum* 'Georgia'. Shoots were developed on solid media and then liquid medium was added into the same vessels in order to form bulblets. The addition of liquid medium stimulated the formation and growth of bulblets compared with vessels that had no liquid media added. Liquid media supplemented with 5 g/L activated charcoal and 250 g/L sucrose formed the most bulblets and bulblets with the largest weight. HAN *et al.* (2004) reported that the addition of liquid medium into the same vessel was a simple and efficient method to increase bulblet formation from shoot clusters without transferring the shoots to new media. This method has great potential to reduce labour costs in the tissue culture of plants.

6.2 MATERIALS AND METHODS

These experiments were carried out to determine the effect of static or shake liquid culture as well as the use of leaf or shoot explants on bulb induction. Pieces of leaf material or 5 individual shoots, from previously established cultures, were placed in 100 ml Erlenmeyer flasks containing 40 ml liquid MURASHIGE and SKOOG (1962) medium. The medium was supplemented with 100 mg/L myo-inositol, 30g/L sucrose and no agar was added. No hormones were added. The pH of the medium was adjusted to 5.8 with diluted NaOH before autoclaving at 121 ℃ and 103 kPa for 20 min. Eight Erlenmeyer flasks containing leaf explants and 8 containing shoots were placed on a rotary shaker at 100 rpm, while 16 flasks (8 containing leaves and 8 containing shoots) were placed on a shelf in a growth room. Leaf explants (5 pieces per jar) were also placed in culture bottles (6 cm diameter, 10 cm high) on 40 ml solid MS. The above MS basal medium was used but it was solidified with 8 g/L agar. The cultures were grown at 25℃ ± 2 ℃ under a 16 h pho toperiod. The growth room had

Osram L58W/640 cool white florescence light bulbs with an average light intensity of 74.4 µmol/m^2/s.

After 3 months bulblets were removed, and bulb mass and diameter were recorded. Data collected were subjected to one-way analysis of variance (ANOVA). Where there is significant difference (p=0.05), the means were separated using Duncan Multiple Range Test (DMRT). Data analysis was done using SPSS version 15.0.

6.3 RESULTS

Leaf explants and shoots in liquid shake culture turned brown in colour and showed no response or growth (Figure 6.1). Leaf explants and shoots in the static Erlenmeyer flasks remained green and shoot, bulb and root growth was observed. The leaf explants produced small protuberances which developed into bulbs. The shoots however did not produce a large number of bulbs, instead the individual shoots swelled and developed into bulbs (Figure 6.1 F). The bulblets that developed on the leaf explants were smaller in size (2 to 5 mm) compared with the bulblets that developed from the shoots, (4 to 8 mm). Solid medium produced better results than liquid shake culture but compared with the liquid static culture, results were not as good (Figure 6.2). All bulblets showed good root growth but the shoots had the longest and highest number of roots. These results are not shown.

The product value was calculated to determine which was the optimum treatment. Static shoots had the highest value, 138.19, as the largest and heaviest bulbs were produced from the shoots. Static leaves had the next highest value of 24.96.

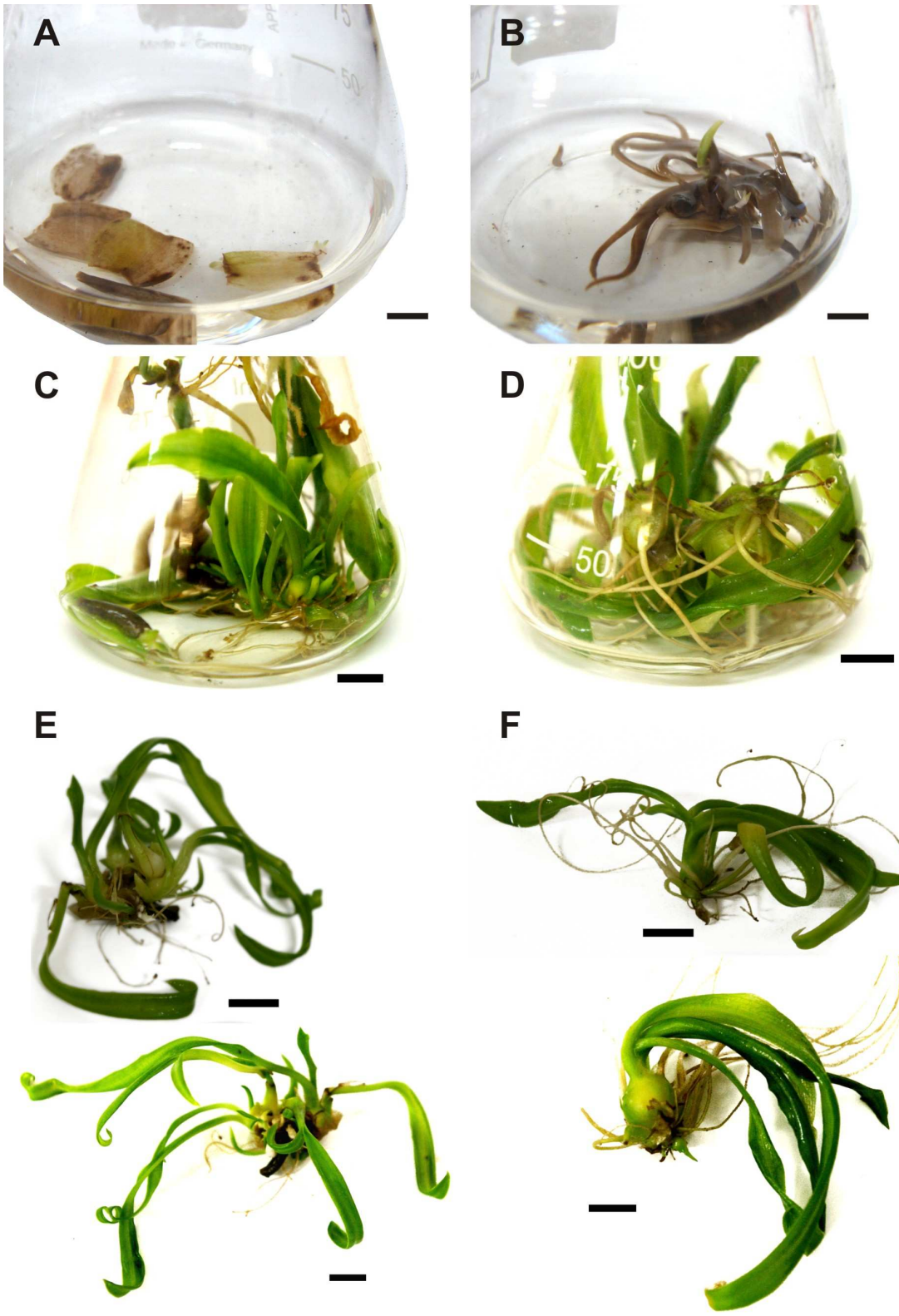


Figure 6.1: **Leaf and shoot explant growth of *E. zambesiaca* from shake and static liquid culture. A = shake leaves B = shake shoots C= static leaves D = static shoots E = individual leaf explant from static culture F = individual shoot from static culture. Bar = 1 cm.**

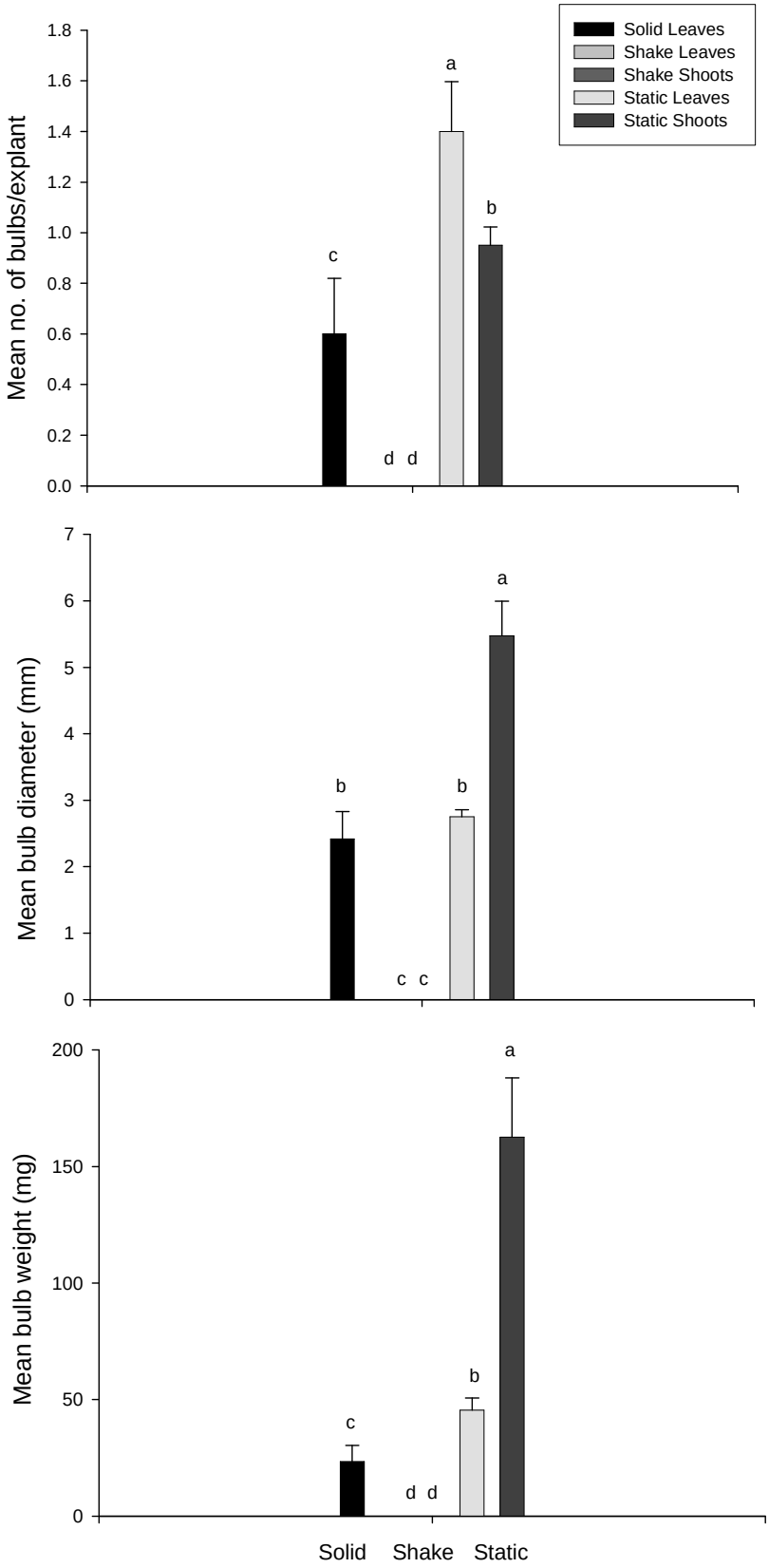


Figure 6.2: The effect of different liquid culture treatments on bulblet induction on leaf explants and shoots of *E. zambesiaca*. Bars bearing different letters are significantly different, P ≤ 0.05.

Table 6.1: Product value of the different types of culture treatments showing which is the optimum treatment.

Type of culture and explant	Product value
Solid leaves	12.29
Shake leaves	0.00
Shake shoots	0.00
Static leaves	24.96
Static shoots	138.19

6.4 DISCUSSION

A number of plant tissue culture techniques have been developed for propagating ornamental species and some of these techniques are being used for large scale commercial propagation. One of these techniques is liquid culture.

BERGOÑÓN *et al.* (1992) showed that liquid shake culture is a simple, easy, rapid and economical method for the propagation of bulbous plants. This method can be optimized by selecting an adequate medium and time of induction as well as using bioreactors to maximize the production. Large quantities of plants of medicinal, ornamental and ecological interest can be obtained.

As plants formed in liquid culture were larger, it may be possible to harvest *E. zambesiaca* bulblets in a shorter period of time using liquid culture compared to explants grown on solid MS medium. Leaf and shoot explants grown in liquid static culture produced 1.4 and 0.95 bulbs per explant respectively compared with 0.60 bulbs per explant grown on solid medium (Figure 6.2). Liquid shake culture was unsuccessful as no growth was observed for both the leaf explants and shoots. This could have been a result of shearing or possibly leaching. These results contradict the findings of TAKAYAMA *et al.* (1991), ILAN *et al.* (1995) and NHUT *et al.* (2004), who reported liquid shake culture to be the better system for the propagation of shoots and bulbs of *Hyacinthus orientalis, Brodiaea* and *Gladiolus* respectively.

VARSHNEY *et al.* (2000) developed a protocol for the *in vitro* mass propagation of two cultivars of Asiatic lily hybrids. In liquid shake cultures, the bulb scale clusters turned brown, while in contrast, stationary cultures resulted in better scale proliferation. These results are in agreement with those found for bulblet formation of *E. zambesiaca*. Therefore, it appears that liquid stationary culture is more effective than liquid shake culture with regards to bulblet induction in *E. zambesiaca*.

Liquid static culture was not only a useful technique for the production of bulblets but also for the enhancement of bulblet growth and size. Mean bulb diameter and weight was considerably higher when leaf explants and shoots were placed in liquid culture compared to those grown on an agar solidified medium. Leaf explants in static liquid culture produced bulbs with a mean diameter of 2.75 mm and a mean weight of 45.38 mg. Bulblets that developed from shoots in static liquid culture were even bigger with a mean diameter and weight of 5.47 mm and 162.57 mg. Bulblets that formed on leaf explants on solid MS were considerably smaller with an average diameter of 2.42 mm and mean weight of 23.40 mg. Liquid culture can thus be used to improve bulblet growth. This was reported by MORÁN *et al.* (2003) who found that liquid culture with 30 or 60 g/L sucrose can be used to accelerate biomass gain and the bulbing of small-sized shoots for both *C. clavatus* and *C. spiralis.* It could therefore be beneficial to place bulbs in liquid culture for a couple of weeks to increase bulb growth prior to transplanting to soil. This will increase the survival rate of bulblets *ex vitro*. This method could be useful for the commercialization of *E. zambesiaca* plants but requires further investigation.

6.5 SUMMARY

- Shake liquid cultures produced no bulblets
- Leaf explants in static cultures produced the greatest number of bulblets
- Shoots in static cultures produced the largest bulblets, with the heaviest weight and widest bulb diameter

Chapter 7
ACCLIMATIZATION

7.1 INTRODUCTION

Acclimatization is the physiological adaptation of a plant to changes in climate or environment, such as light and temperature. Acclimatization is an important stage in micropropagation, however, many micropropagted plants do not often survive the transfer from *in vitro* conditions to the greenhouse or field environment. Plants propagated *in vitro* are exposed to an environment that provides minimal stress and optimum conditions for multiplication (HAZARIKA, 2003). They are developed on media containing sugar and nutrients, which allows for heterotrophic growth, under low light intensities, aseptic conditions and high levels of humidity. Under such high levels of humidity plants develop anatomical features such as a low deposition of surface wax, abnormal stomata and a non-continuous cuticle. These anatomical features and growth environment contribute to a 'culture-induced phenotype'. Most often these *in vitro* grown plants cannot survive the transfer to *ex vitro* conditions (HAZARIKA, 2003). Therefore, when developing a micropropagation protocol, it is important that a "hardening-off" stage be included in the study. TAYLOR & VAN STADEN (2001c) looked at the effect of either vermiculite or perlite medium on the acclimatization of *Eucomis* species and showed that vermiculite was better for the acclimatization of *E. zambesiaca* plantlets.

7.2 MATERIALS AND METHODS

After *E. zambesiaca* bulblets were successfully grown *in vitro,* they were transferred to trays containing vermiculite and placed in a misthouse. After 2 months the plantlets were transplanted to pots containing a sand:soil mixture of 1:1 and moved into a greenhouse.

7.3 RESULTS

All the *in vitro* grown bulblets that were placed in the misthouse grew in size and formed leaves and roots. After removal from the misthouse and transplantation, some of the bulbs lost their leaves and did not survive. Overall, there was an 80-90% survival rate.

Figure 7.1: *E. zambesiaca* bulblets after 2 months in the misthouse.

7.4 SUMMARY

- Bulblets from *in vitro* tissue culture were successfully acclimatized and transplanted for further growth.
- A protocol for the mass bulblet production of *E. zambesiaca* has, therefore, successfully been established.

Conclusions

Micropropagation is the biotechnological tool that is most frequently used for conservation of threatened plant species and to provide stocks for the horticultural industry. By controlling the *in vitro* conditions, optimal conditions for the production of secondary metabolites, plantlet regeneration and growth can be established. Therefore, micropropagation may be used for rapid, year-round, mass multiplication of ornamental geophytes.

Temperature is a major factor in the tissue culture environment and plays a pivotal role in plant growth and morphogenesis. A temperature of 20 ℃ was found to produce the best results from leaf explants of *E. zambesiaca*. An average of 2.6 bulblets formed per explant and the mean bulblet weight per jar was 720 mg. It has been reported that maximum success from tissue culture may only be achieved when the precise temperature needs of a plant are fulfilled.

From previous work and even this study, it can be seen that light and specifically the three parameters of light (duration, quality and intensity) have a large affect on *in vitro* plant tissue culture. NORTON *et al.* (1988) and MICHALCZUK and MICHALCZUK (2000) reported that the use of monochromatic light can improve the efficiency of micropropagation of plants as well as control shoot and root length. Bulb induction on *E. zambesiaca* leaf explants occurred under the photoperiods: continuous light, 16 h photoperiod and 8 h photoperiod however, continuous darkness completely inhibited growth. The optimum photoperiod was 8 h light as it produced the highest number of bulblets per explant (1.38) and had the largest bulbs with an average diameter and weight of 3.38 mm and 42.05 mg respectively. Therefore, the use of light is likely to be a valuable alternative to manipulate plant growth and in so doing, it may eliminate the use of expensive growth regulators.

Changing the carbohydrate in the culture medium to fructose, resulted in an improvement in the number of bulblets produced per explant and an increase in bulblet size. Fructose at a concentration of 30 g/L produced 1.18 bulblets per leaf

explant compared with 0.60 bulblets that formed on the standard sugar, 3% sucrose. Combinations and various concentrations were also tested. Fructose (3%) and a combination of glucose and fructose at a 2:1 ratio produced good sized bulbs. Mean bulb diameter for the two treatments were 3.39 and 3.36 mm, while the mean bulb weights were 56.61 and 46.27 mg respectively. Generally, as the carbohydrate concentration increased, bulblet regeneration and development decreased. The optimum carbohydrate and concentration may vary among plant species with some plants needing a specific carbohydrate while others need higher or lower concentrations.

Plant growth regulators are essential for the *in vitro* propagation of most plants however, growth regulators cause different reactions in different species and cultivars even at different seasons. Therefore, it is not possible to say which growth hormone and concentration will produce the desired results. With *E. zambesiaca,* 1 mg/L IBA produced maximum results. Bulblets were biggest at this concentration with a mean diameter of 4.36 mm and an average weight of 79.07 mg. Other auxins tested such as 2,4-D, IAA and NAA also produced good results compared with the cytokinins. There was a trend that as the hormone concentration increased, bulblet initiation and growth decreased.

Liquid cultures provide a system for rapid growth and multiplication of plants *in vitro*. Liquid shake and liquid static cultures were tested and liquid static cultures were found to produce better results than liquid shake cultures. Liquid shake cultures produced no results while bulblet, leaf and root growth was of high quality in liquid static cultures. Therefore, these experiments showed that liquid static culture allows for the production of *in vitro* storage organs in a shorter period of time.

A protocol has successfully been established for *Eucomis zambesiaca,* a medicinal geophyte with an attractive ‘pineapple’ inflorescence. Successful micropropagation from leaf explants has proven that leaf explants can be used as an alternative explant source to produce bulblets readily. It is therefore recommended that leaf

explants should be grown on media supplemented with 30 g/L fructose, 1 mg/L IBA and under a 8 h photoperiod at 20 ℃. Bulblets can then be placed in static liquid culture to increase bulblet size. Rapid rates of propagation using this protocol allows for the fast and economic mass propagation of *Eucomis* plants for the use in commercial or conservation efforts.

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