In vitro propagation of *Dierama erectum*

Submitted in fulfilment of the requirements for the degree of

MASTER OF SCIENCE

By

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STUDENT DECLARATION

In vitro* propagation of *Dierama erectum

I, Motselisi Jane Koetle, 208523555 *(Student Number)*

declare that :

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

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DECLARATION BY SUPERVISORS

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

Student's Full Name: Motselisi Jane Koetle

Student Number: 208523555

Thesis Title: *In vitro* propagation of *Dierama erectum*

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.

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SUPERVISOR: PROFESSOR J VAN STADEN

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PUBLICATION IN PREPARATION

KOETLE, M.J., FINNIE, J.F., VAN STADEN, J. *In vitro* regeneration of *Dierama erectum*

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LIST OF ABBREVIATIONS

2,4-D ……..	2,4-Dichlorophenoxyacetic acid
AC ……..	Activated charcoal
ANOVA ……..	Analysis of variance
BA ……..	N^6-Benzyladenine
C_a ……..	Chlorophyll *a*
C_b ……..	Chlorophyll *b*
C_{x+c} …….....	Total carotenoid
DMRT…….....	Duncan's Multiple Range Test
DNA…….....	Deoxyribonucleic acid
$HgCl_2$ ……	Mercuric chloride
IAA ……	Indole-3-acetic acid
IBA ……	Indole-3-butyric acid
IUCN ……..	International Union for the Conservation of Nature
KIN..……	Kinetin
LSD ……	Least Significant Difference
MS ……..	Murashige and Skoog (1962) medium
mRNA ……	Messenger ribonucleic acid
*m*T ……..	*meta*-Topolin
NAA……	Naphthaleneacetic acid
NaOCl ……	Sodium hypochlorite
PGR ……...	Plant growth regulator
PVP ……	Polyvinylpyrrolidone
UV ……..	Ultra violet
Z ……	Zeatin

ABSTRACT

Dierama is a genus of plants with a potential to be developed as ornamental plants. It falls under the Iridaceae family and comprises of 44 species. *Dierama erectum* Hilliard, an attractive species with horticultural potential is mainly found in rough wet grasslands. Its corms are used for enemas and treating stomach ailments in southern African traditional medicine. Due to its habitat transformation by afforestation and the exploitation of its underground parts (corms) in traditional medicine, this plant is among the most vulnerable and rare species within its genus. Seed parasitism by *Urodon lilli* also hampers its conventional propagation.

The increase in demand for ornamental and medicinal plants increases pressure on wild plant populations. Micropropagation is a useful tool for clonal propagation of plants as it does not only help in alleviating pressure on wild plants but an effective micropropagation protocol could also provide a foundation for plant genetic transformation, which could result in the development and introduction of new ornamental varieties into commercial markets. This research was aimed at developing a micropropagation protocol for *D*. *erectum* to ensure readily available source material for medicinal and horticultural use as well as serving as an alternative for its conservation.

Seed decontamination and germination were successful when 0.2% $HgCl_2$ or 2.5% NaOCl + 1% Benlate® were used. However, for safety reasons, 2.5% NaOCl + 1% Benlate® was used in all subsequent experiments. The shoot regenerative capacity

of leaf, hypocotyl and root explants obtained from *in vitro* germinated seedlings was evaluated by culturing them individually on MS medium supplemented with various concentrations of BA. Only hypocotyl explants produced adventitious shoots. Since no shoots or callus was produced from leaf and root explants, hypocotyl explants were used in the development of a micropropagation protocol.

Different types and concentrations of cytokinins (BA, *m*T, KIN and Z) with or without NAA were evaluated for their effect on adventitious shoot production. Maximum shoot number per explant (4.20 ±0.51) was obtained in MS medium supplemented with 1.0 µM Z after 8 weeks. This was followed by a combination of KIN (2.0 µM) and NAA (0.5 µM) resulting in a production of 3.67 ± 0.81 shoots per explant. For BA treatments, the highest shoot multiplication (3.20 ± 0.22 shoots per explant) was achieved when 2.0 µM was combined with 1.0 µM NAA. *m*T gave maximum shoot production (3.09 ± 0.99 shoots per explant) when 2.0 µM *m*T was combined with 2.0 µM NAA.

The effects of photoperiod and light intensity were investigated for the purpose of optimizing shoot multiplication. An average of 12.73 ± 1.03 shoots per explant were obtained after 8 weeks from shoots grown in 16 h light at a 100 µmol m^{-2} s^{-1} light intensity. The 24 h light treatments and a light intensity lower than 100 µmol m^{-2} s^{-1} negatively affected growth and regeneration of *D. erectum*. These results highlighted the need for evaluating environmental conditions when developing micropropagation protocols.

Corm induction experiments were done with the intention of facilitating acclimatization of *D. erectum ex vitro*. Various concentrations of ancymidol, activated charcoal and sucrose did not promote *in vitro* corm formation, thus auxins (IAA, IBA and NAA) were tested for their efficiency in rooting. Plants rooted successfully after 8 weeks on MS medium supplemented with 1.0 µM IBA, yielded the longest roots (4.63 ± 0.70 cm) and an average root number of 2.73 ± 0.40. All NAA treatments resulted in stunted roots.

Plants grown *in vitro* were potted in trays containing a 1:1 ratio of soil: vermiculite and placed in the mist house for 2 weeks. They were then transferred to the greenhouse for further acclimatization. After 2 months, plants had formed corms. The largest corms (0.45 ± 0.026 cm in diameter) were found in plants pre-treated with 0.5 µM IBA. Maximum plant survival percentage (73%) was also associated with this treatment. A successful micropropagation system for *Dierama erectum* was therefore developed. The utilisation of this protocol can yield about 15137 plants from one explant in a year. This will expand our existing knowledge about micropropagation of plants in the genus *Dierama* and will be useful in the conservation of this species.

CHAPTER ONE: INTRODUCTION

1.1 PLANTS AND MICROPROPAGATION

The human population growth rate is increasing, leading to severe pressure on the demand for ornamental and medicinal plants. This pressure results in species decimation arising mainly from destructive harvesting and destruction of plant habitats and ecosystems **(FAY, 1992)**. Destructive harvesting often involves the removal of whole plant parts and underground parts (bulbs, corms, rhizomes and roots) that are essential for plant regeneration and survival. **SARASAN et al. (2006)** reported that during the period 1996 to 2004, a total of 8321 plant species were added to the International Union for the Conservation of Nature and Natural Resources (IUCN) red list of threatened species. It is further reported that during this period, there was an increase of over 60% in the number of plants regarded as critically endangered.

In Africa, the majority of the population uses traditional medicine because of its affordability. For this reason, an increasing trade in medicinal plants is threatening the survival of many species **(CUNNINGHAM, 1993)**. While demand for medicinal plants exceeds supply, provision of alternative sources of medicinal plants by encouraging their cultivation will go a long way in reducing the heavy dependence on wild populations. Large scale production through *in vitro* propagation of plants provides a means of ensuring availability of medicinal and horticultural plants in the market **(WOCHOK, 1981)**.

1.2 THE FAMILY IRIDACEAE

The family Iridaceae contains 65 genera and approximately 2025 species **(ROURKE, 1996)**. It is more widely distributed in South Africa than elsewhere. Most of the genera are deciduous, sprouting from underground corms with the advent of the wet season, flowering, then dying back and becoming dormant during the dry season. *Moraea, Hesperantha, Romulea, Geissorhiza, Gladiolus* and *Babiana* are among the largest genera which are common in the Western Cape, South Africa. They flower in autumn, winter and spring **(ROURKE, 1996)**.

The family Iridaceae is relatively easy to recognize amongst monocotyledons; the flowers have only three stamens and the ovary is inferior. *Dierama* falls within the subfamily, Ixioideae which comprises more than half of the family. The Ixioideae is characterized by differences between the upper and lower petals; these differences are brought about by the change from radial symmetry to bilateral symmetry. The anthers are grouped together on the upper side of the flower making one pollen source for pollinators. The Ixiodeae is also predominantly comprised of corm-forming plants rather than the rhizome-forming plants as is the case with other subfamilies within the Iridaceae **(HILLIARD and BURTT, 1991)**.

1.3 THE GENUS *DIERAMA*

Dierama is commonly known as hair-bells. It is a genus of the Iridaceae family, noted for its tall flower stalks that divide at the top into a number of pendulous flowering branches making the bell-shaped flowers wave in the slightest breeze **(HILLIARD and BURTT, 1991)**. In South Africa, the genus has its main centre of endemism south of the Limpopo, with KwaZulu-Natal having the greatest number of species **(ROURKE, 1996)**. In KwaZulu-Natal and Mpumalanga, clumps of *Dierama* are found in moist grasslands. Many *Dierama* species are found mainly in the summer rainfall region of eastern southern Africa, but overall distribution extends from Knysna in the Western Cape, South Africa, to Ethiopia **(ROURKE, 1996)**. In the tropics, *Dierama* species are largely montane, whereas in KwaZulu-Natal and in the Eastern Cape, they are naturalised from sea level on stabilized sand flats to the top of the Drakensberg escarpment at about 3000 m **(ROURKE, 1996)**.

Some *Dierama* species are prominent in the foothill flora of Lesotho. Species like *D. igneum* Klatt and *D. robustum* N.E.Br. are found in the Senqunyane River Valley, and Jordan Valley in the Leribe district **(GUILLARMOD, 1971)**. Other species like *D. igneum* Klatt, *D. jacundum* Hilliard, *D. robustum* N.E.Br. and *D. trichorhizum* (Baker) N.E.Br. are found in the lowland zone, foothill zone and mountain zone while *D. dracomountanum* Hilliard has only been recorded once in 1977 between the Mafeteng and Mohale's Hoek districts of Lesotho **(GOLDING, 2002)**.

Despite their wide geographic and altitudinal range, *Dierama* species are restricted to moist grassland habitats. The plants are evergreen, thus they not only require rain during the period of growth, but they need sufficient moisture all year round **(HILLIARD and BURTT, 1991)**.

Figure 1.1: Seed collection from *Dierama erectum* plants in Mt. Gilboa (**A**). The inflorescence (**B**).

1.4 *Dierama erectum* Hilliard

1.4.1 Morphology

Dierama erectum is found in rough wet grasslands near streams, growing to a height of about 1.65 m when flowering. It has an erect inflorescence with about

10 flowers on each branch. The flowers have a light magenta-pink colour with “eyes” at the base of each petal (**Figure 1.1B**). They are erect, crowded and have robust bracts **(HILLIARD and BURTT, 1991; POOLEY, 1998)**.

1.4.2 Medicinal use

The Sotho use *Dierama erectum* as a strong purgative enema and to cure venereal diseases **(WATT and BREYER-BRANDWIJK, 1962)**. It is also used as a remedy for stomach ailments **(HILLIARD and BURTT, 1991)**. In South Africa, corms are placed in seed-gourds as fertility charms to ensure a good harvest **(HUTCHINGS et al,, 1996)**.

1.4.3 Horticultural use

Dierama species are attractive and have horticultural potential. **SCOTT-SHAW (1999)** elaborated on the high economic and horticultural potential of *D. erectum* as it is large in size and has erect flowers. The successful introduction of *Dierama* species as garden plants has been recorded as early as 1825 in Britain and France, but there is no precise record of *Dierama* species being grown as ornamentals in their native Africa **(HILLIARD and BURTT, 1991)**.

1.4.4 Conservation status

There are about 11 endemic *Dierama* species recorded in KwaZulu-Natal. Five out of eleven species namely, *D. erectum* Hilliard, *D. luteoalbidum* Verdoorn, *D.*

nixonianum Hilliard, *D. pallidum* Hilliard and *D. pumilum* N.E.Br. are regarded as vulnerable in their natural habitats **(SCOTT-SHAW, 1999)**. In South Africa, *Dierama erectum* is known only from the Ngome district in northern KwaZulu-Natal **(HILLIARD and BURTT, 1991)**. It is vulnerable, very rare and has a narrow distribution. Its vulnerability is attributed to afforestation which has transformed its grassland habitat **(SCOTT-SHAW, 1999)**.

1.4.5 Propagation

Although *Dierama* species are easily grown from seed, seeds collected from the wild are frequently infested by bruchid beetles (*Urodon lilii*). The female beetle lays its eggs on the outside of the young ovary and the grub burrows through the wall into the ovule. The development of the outer layers into a normal, brown and shining seed coat may not seem to be impeded during early stages of seed development, but when ripe seeds are collected, some of them feel slightly soft to the touch and when opened, are found to contain a beetle-grub. This therefore hampers conventional propagation of *Dierama* species **(HILLIARD and BURTT, 1991)**.

Another form of propagation is by division of clumps and this is done in Spring, but care must be taken when up-lifting the plants from the soil because the roots are very brittle. Divisions are replanted immediately and kept well watered. However, there is a high chance of plant loss through pathogen attack **(DUNCAN, 1996)**. Moreover, plants propagated this way take at least a year to

become well acclimatized to their new environment **(HILLIARD and BURTT, 1991)**.

1.5 THE IMPORTANCE OF MICROPROPAGATION

The impact of human activities on natural ecosystems has become an issue of great public concern. Human activities in certain regions have caused a depletion of many plant species that might have otherwise remained secure in their natural habitats **(WOCHOK, 1981)**. Several options such as seed and vegetative propagation are available to address this problem. One method that has great potential for germplasm preservation is tissue culture **(FAY, 1992)**.

As the demand for medicinal and ornamental plants increases, new varieties must be made available in sufficient quantities in the commercial markets **(GILES and MORGAN, 1987)**. Breeders can introduce genetic variation into ornamentals by application of recombinant DNA technology and mutagenesis. This technology is useful for bringing changes in phenotypic expressions such as corolla and foliage colour, stem length, scent, regulation of flowering and resistance to stressful conditions **(KHAYAT, 1990)**. In this case, micropropagation is useful in shortening the time needed to commercialize new varieties **(GILES and MORGAN, 1987)**. Micropropagation helps in the production of disease-free plants, thus allowing transformation involving disease resistance in plants possible **(HUSSEY, 1980)**. Recalcitrant species can also be rapidly multiplied through tissue culture **(KIM and DE HERTOGH, 1997)**.

1.6 MICROPROPAGATION OF SOME IRIDACEAE SPECIES

There are three potential areas for commercial development of plants belonging to this family: (1) improvement of existing varieties which is an important aspect in plant breeding; (2) finding new uses for known varieties; and (3) research on little known species to develop them into new commercial crops **(ASCOUGH et al., 2008a)**. Tissue culture techniques have been used successfully for propagating some members of Iridaceae. In the review of micropropagation of the Iridaceae, **ASCOUGH et al. (2009a)** observed that protocols have been developed for some members of the genera *Babiana*, *Cipura*, *Crocosmia*, *Crocus*, *Dierama* (*D. latifolium* and *D. luteoalbidum*), *Freesia*, *Gladiolus*, *Iris*, *Ixia*, *Schizostylis*, *Sparaxis* and *Watsonia.* Successful micropropagation in this family has been achieved using explants such as anthers, bulbs, corms, flowers, hypocotyls, leaves, ovaries, plantlets, roots, shoots and twin scales **(ASCOUGH et al., 2009a)**.

More work is required to obtain a better understanding of micropropagation in Iridaceae **(ASCOUGH, 2008)**. Ornamental geophytes like *Dierama* species are little researched and more knowledge of the tissue culture of these species needs to be established as a step towards the development of new cultivars for horticultural markets.

Table 1.1: Iridaceae members propagated through tissue culture

Plant name	**Growth response***	**Reference**
Babiana angustifolia Sweet	S, R, C	**McALISTER et al. (1998)**
Babiana disticha Ker Gawl.	S,R,C	**McALISTER et al. (1998)**
Crocosmia crocosmiiflora Planch	S,R,C	**KOH et al. (2007)**
Crocus almehensis C.D. Brickell	S, C	**EBRAHIMZADEH et al. (1996)**
Crocus sativus L.	S, R, Cm	**PLESSNER et al. (1990)**
Dierama latifolium N.E. Br.	S, R, C	**PAGE and VAN STADEN (1985)**
Dierama luteoalbidum Verdoorn	S, C, Cm	**MADUBANYA et al. (2006)**
Gladiolus dalenii Baker	S, Cm	**DE BRUYN and FERREIRA (1992)**
Gladioulus flanaganii Baker	S, R, Cm	**DIKENS et al. (1986)**
Iris ensata Thunb	S	**SHIMIZU et al. (1999)**
Iris germanica L.	S, R	**SHIMIZU et al. (1999)**
Axia flexuosa L.	S, C	**MEYER and VAN STADEN (1998)**
Schyzostylis coccinea Backh & Harv	S, C	**HUSSEY (1976)**
Sparaxis bicolour Ker Gawl.	S, C	**HUSSEY (1975)**
Watsonia gladioloides Schltr	S, R	**ASCOUGH (2008)**
Watsonia laccata Ker Gawl.	S, R	**ASCOUGH (2008)**
Watsonia lepida N.E. Br.	S, R, Cm	**ASCOUGH (2008)**
Watsonia vanderspuyiae L. Bolus	S, R, Cm	**ASCOUGH (2008)**

* S = shoots, R = roots, C = callus, Cm = corm

1.7 BACKGROUND TO THE RESEARCH PROBLEM

Dierama species like *D. erectum* are ranked vulnerable in the Red Data List because they are rare, showing narrow distribution and a very low abundance in their natural habitats **(SCOTT-SHAW, 1999)**. In addition, conventional propagation of *D. erectum* by seed is hampered by seed parasitism. **SCOTT-SHAW (1999)** indicated that there is a need for propagation of this plant to provide an *ex situ* reserve of propagules.

The establishment of an effective micropropagation protocol in this study is an important step towards introducing new ornamental plants in the family Iridaceae. It does not only provide rapid propagation of disease-free plants, but lays a foundation for other biotechnological applications such as genetic transformation and plant breeding.

1.8 AIMS OF THE STUDY

This study was aimed at developing a micropropagation protocol for *Dierama erectum* so as to ensure readily available source material for medicinal and horticultural use thereby reducing pressure on collection from wild populations. The specific objectives were:

- Establishing an effective decontamination protocol for seeds;
- Determining the effects of different types and concentrations of plant growth regulators (PGRs) on shoot multiplication of *D. erectum;*

- Investigating the effects of photoperiod and light intensity on growth of *D. erectum;*
- Determining the effects of different concentrations of ancymidol, activated charcoal and sucrose on corm induction;
- Investigating the effects of various types and concentrations of auxins on rooting; and
- Developing an effective post-flask management of *in vitro* regenerated plants.

1.9 GENERAL OVERVIEW OF THE THESIS

This thesis is arranged in four chapters. The current Chapter serves as a general introduction. Chapter Two is the review of literature on the role of plant growth regulators in micropropagation as well as effects of other media additives and environmental factors (photoperiod and light intensity) on micropropagation of *Dierama* species. Chapter Three gives the details involved in developing a micropropagation protocol for *D. erectum*. Chapter Four provides insights into corm induction, rooting and acclimatization of this species. Conclusions stemmed from this study, as well as recommendations, are given in Chapter Five.

CHAPTER TWO:
LITERATURE REVIEW

2.1 INTRODUCTION

Some plant cells are totipotent and retain their embryonic competence. This means a single cell has the ability to regenerate into an entire plant **(FENNELL et al., 2001)**. In plant tissues, competent cells are those which respond to external signals to enter a specific developmental pathway **(MERCIER et al., 2003)**. Plant growth occurs through cell division and organs are generated either directly or indirectly through meristems which have cells that are capable of dividing **(ASCOUGH et al., 2009b)**.

As a result of cell division in tissue culture, adventitious shoots are formed first, followed by adventitious root formation. Shoot organogenesis *in vitro* is a unique process because shoot meristems are induced from somatic cells and not from embryonic cells. It is a process involving: (1) response of somatic cells to plant growth regulators (PGRs), (2) cell division of the responding cells and (3) induction and development of new shoots **(MERCIER et al., 2003; GABA, 2005)**. Applications of PGRs help by speeding up the timing of the cell cycle and to program the resulting cells with a new developmental fate **(PIERIK, 1987)**.

In the history of micropropagation, monocotyledonous plants such as those in the family Iridaceae were regarded as difficult to establish *in vitro* **(HUSSEY, 1975)**. The speed of shoot induction in micropropagation varies considerably

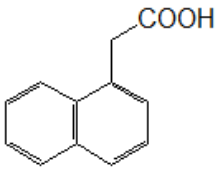
from species to species even within the same family. This characteristic is entirely dependent on factors such as explant type, orientation, growth medium type, plant age, collection time of the plant material and the environment to which the explants are exposed.

2.2 PLANT GROWTH REGULATORS IN MICROPROPAGATION

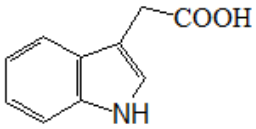
In vitro growth and development is affected by a number of factors including: the genetic make-up of the plant, nutrients (water, macro-elements and micro-elements and sugars), physical growth factors (light, temperature, pH, oxygen, carbon dioxide concentrations) as well as some organic substances such as plant growth regulators (PGRs) and vitamins **(PIERIK,1987)**. PGRs are low-molecular weight natural products that act at very low concentrations (micromolar or even lower) to regulate all physiological and developmental processes in plants **(BAJGUZ and PIOTROWSKA, 2009)**. These compounds include auxins, cytokinins, abscisic acid, gibberellins, ethylene, polyamines, jasmonates, salicylic acid and brassinosteroids **(SRIVASTAVA, 2002)**. They coordinate plant development at all levels; from cellular levels to organs and finally, the whole plant. Some PGRs are synthesised by plant cells for their own consumption whereas others are synthesized in one organ and transported to other plant parts for specific actions. PGRs produced by the plant itself are referred to as endogenous PGRs. Those that are synthesised and applied to intact plants and tissue cultures are known as exogenous PGRs **(GABA, 2005)**.

2.2.1 Auxins

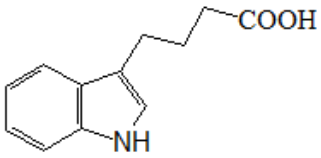
Auxins are known to affect many processes in plants including cell elongation, apical dominance and adventitious root formation **(SRIVASTAVA, 2002; GABA, 2005)**. The most commonly used auxins in micropropagation include indole-3-acetic acid (IAA), indole-3-butyric acid (IBA), 1-naphthaleneacetic acid (NAA) and 2,4-dichlorophenoxyacetic acid (2,4-D) **(PIERIK,1987)**. When auxins are applied at high concentrations, they may induce callus formation and somatic embryogenesis. In addition, they can be toxic because they stimulate ethylene production, which may cause growth inhibition **(GABA, 2005)**.



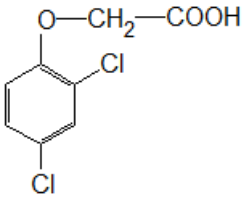
Naphthalene-1-acetic acid (1-NAA)



Indole-3-acetic acid (IAA)



Indole-3-butyric acid (IBA)



2,4-Dichlorophenoxyacetic acid (2,4-D)

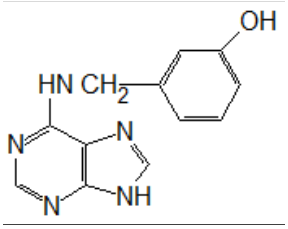
Figure 2.1: Structural formulae for selected auxins.

2.2.2 Cytokinins

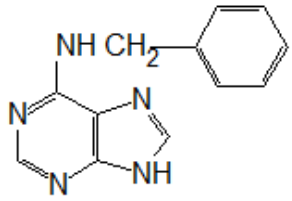
Cytokinins are a class of PGRs that are important at all phases of plant development from seed germination to senescence. At a cellular level, they induce gene expression and promote mitosis **(BAJGUZ and PIOTROWSKA, 2009)**. In combination with auxins, they regulate the ratio of shoot bud and root growth in stem cuttings. In intact plants, they regulate apical dominance and lateral root initiation **(SRIVASTAVA, 2002)**. Cytokinins are required for vascular morphogenesis in the roots and root formation **(MALÁ et al., 2009)**. They also retard senescence and chlorophyll degradation in aging leaf tissues **(SRIVASTAVA, 2002)**. Excessive concentrations of cytokinins applied to the growth medium may cause growth of many small shoots which usually fail to develop **(GABA, 2005)**. The differences in cytokinins are related to their structural features such as the side chain attached to the adenine group, their conjugation with sugars and phosphorylation **(GALUSZKA et al., 2008)**. These structural variations are responsible for observable biological activities of cytokinins **(SAKAKIBARA, 2005)**.

Auxin-cytokinin interaction is an important aspect in micropropagation. The appropriate balance between the two is required to initiate plant growth **(GASPAR et al., 1996)**. For example, in micropropagation of pineapple, experiments demonstrated that an exogenous hormonal balance between NAA (1.0 mg L^{-1}) and BA (2.0 mg L^{-1}) was the best for protuberance induction on the pineapple leaf bases **(MERCIER et al., 2003)**. Even so, relative proportions of auxins and cytokinins do not always produce typical responses, for example: (1)

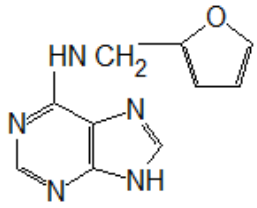
Shoot proliferation in some species may be stimulated by presence of auxin together with cytokinin. (2) Tissues from monocotyledons can be induced to form callus or plantlets by culture in altered levels of auxin alone and cytokinins may be non-essential **(GEORGE et al., 2008)**. This was found to be true in the study conducted by **PAGE and VAN STADEN (1985)**, where corm explants of *Dierama latifolium* produced shoots when cultured on a medium containing 0.5 mg L^{-1} NAA. Shoot induction from these explants was not enhanced by addition of BA to the growth medium.



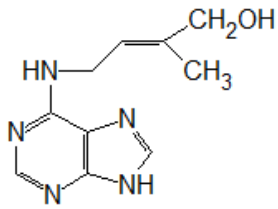
meta-Topolin (*m*T)



N^6- Benzyladenine (BA)



Kinetin (KIN)



trans-Zeatin (Z)

Figure 2.2: Structural formulae for selected cytokinins.

2.2.3 Growth retardants

Growth retardants act as plant growth regulator antagonists by blocking their synthesis or action. Growth retardants such as ancymidol, an inhibitor of gibberellin biosynthesis, are used to control morphological and physiological disorders, shoot elongation and meristematic cluster induction in liquid-cultured plants **(CHEN and ZIV, 2001)**. In the study conducted by **ILAN et al., (1995)**, a decrease in biomass in the presence of growth retardants was observed and this was attributed to prevention of leaf growth by these substances. Bulbs were produced from *Crinum* twin scales when the growth medium was supplemented with 1.25 mg L^{-1} ancymidol **(SLABBERT et al., 1993)**. Corm formation in *Dierama luteoalbidum* occurred after three months and more corms were produced when 5 and 10 mg L^{-1} paclobutrazol was added to the medium **(MADUBANYA, 2004)**.

2.3 EFFECTS OF MEDIA ADDITIVES ON MICROPROPAGATION

Nutrients added in the tissue culture medium are essential for the growth and development of the plant. The type of growth media used depends on the species to be cultured. Some species are sensitive to high salt concentrations and have different requirements for nutrients *in vitro* **(GEORGE et al., 2008)**. It may be necessary to determine concentrations of nutrients or other media additives optimum for *in vitro* growth of plants to achieve successful micropropagation protocols.

2.3.1 Carbohydrates

Carbohydrates are important in plant life. They are produced during photosynthesis then transported to sink tissues, channelled to respiration or converted into storage compounds such as lipids, starch, sucrose or fructans **(LORETI et al., 2001)**. Carbohydrate biosynthesis and its metabolism is an important characteristic to be considered during storage organ development in geophytes **(CHEN and ZIV, 2001)**.

Sucrose is generally regarded as a standard carbohydrate in plant tissue culture **(EVANS et al., 1981; DEBNATH, 2005)**. However, some carbohydrate sources such as maltose have proved to be better than sucrose in terms of their effect on embryos and plant development from barley microspores **(SCOTT and LYNE, 1994)**. Those authors have observed that barley microspores cultured in the presence of sucrose, glucose and fructose died within three days of culture, while with maltose concentrations above 20 mM, there was an increase in the viability of barley microspores.

DANTU and BHOJWANI (1995) established the validity of sucrose as a carbohydrate source and found optimum concentration for corm induction in *Gladiolus* to be 6% w/v. Addition of 5-8% w/v sucrose in the growth medium enhanced corm formation in *Brodiaea* species **(ILAN et al., 1995)** while in another experiment conducted by **VISHNEVETSKY et al. (1997)**, bulbs of *Nerine sarniensis* grew best in media containing 6% w/v sucrose. It can therefore be assumed that a carbohydrate supply during tissue culture may

contribute to the growth of storage organs such as corms and bulbs **(ASCOUGH, 2008)**. It is also important to note that the success or failure of a carbohydrate to induce storage organs may be dependent on the type and concentration used in the growth medium.

2.3.2 Activated charcoal

Activated charcoal (AC) is a tasteless material which is composed of carbon arranged in a quasigraphitic form in small particle sizes **(THOMAS, 2008)**. It is manufactured from elementary carbon by removal of all non-carbon impurities and the oxidation of the carbon surface. It has a very fine network of pores and a large surface area that gives it a high adsorption capacity **(THOMAS, 2008)**.

The use of AC may bring about the success or failure of a given tissue culture experiment **(PAN and VAN STADEN, 1998)**. Some effects of charcoal on *in vitro* cultures include provision of some degree of darkness during *in vitro* culture and the adsorption of undesirable or inhibitory substances such as phenolics. Phenolics often have some inhibitory effects and should be avoided in *in vitro* environments. Date palm tissue cultures have been reported to release some deeply coloured substances in the growth medium, and this inhibited growth *in vitro*; this problem was overcome by adding charcoal to the nutrient medium **(WANG and HUANG, 1976)**. AC has been reported to help in adsorption of PGRs. It is used for the adsorption of toxic plant metabolites and high concentrations of growth regulators in both liquid and solid media **(PAN and VAN STADEN, 1998)**.

AC is also used when solving some physiological problems encountered in tissue culture. For example, addition of AC reduced chlorosis in Mexican redbud (*Cercis canadensis* var. *mexicana*) when added to the growth medium **(MACKAY et al., 1995)**. Explant browning in tissue culture is a serious problem. To solve this problem in raspberry (*Rubus idaeus* L.), **WANG et al. (2005)** fortified MS medium with AC, ascorbic acid, citric acid and polyvinylpyrrolidone (PVP). It was found that cultures grown in a 0.25 g L^{-1} AC-supplemented medium had the least browning.

In some plants, the induction of storage organs is highly affected by AC in the medium. In *Lilium* species, a significantly high percentage of large bulblets were obtained when 0.04 g L^{-1} AC was added in the medium **(BACCHETTA et al., 2003)**. **MOHAMED-YASSEEN et al. (1993)** observed that bulblets were induced in *Allium sativum* on MS medium supplemented with 5 g L^{-1} AC.

2.4 ENVIRONMENTAL FACTORS IN MICROPROPAGATION

2.4.1 Photoperiod

Light conditions play an important role in micropropagation systems. They influence micropropagation through three parameters: photoperiod, intensity and spectral quality **(ECONOMOU and READ, 1987)**. Many studies have demonstrated different ways in which photoperiod affects different plant species but generally, the most favourable growth condition for most plants is a 16-8 h light-dark cycle **(MORINI et al., 1991)**. Photoperiod affects various

developmental processes such as rooting, stem elongation **(RAMANAYAKE et al., 2006)** and stomatal regulation **(STADLER et al., 2003)**.

The length of photoperiod affected growth of *Alocasia amazonica* significantly. Higher fresh and dry weight indicated that this plant requires a shorter photoperiod (8 h light and 16 h dark cycle) for its best vegetative growth **(JO et al., 2008)**. Photoperiod also affects *in vitro* storage organ formation. **ASCOUGH (2008)** outlined three categories of plant responses to photoperiod in terms of storage organ formation: (1) Storage organ induction inhibited by continuous darkness, (2) production of storage organs in darkness and (3) induction of storage organs in both light and continuous darkness. All these factors are dependent on the plant species under study.

2.4.2 Light intensity

Controlled light intensity is one of the most effective ways of achieving higher and more stable yields from tissue culture. Plants have different responses to light intensity in tissue culture. These responses may be due to differences in plant anatomy, morphology or their physiology. For instance, **TAPINKAE and TAJI (2000)** found that the dry weight of *Anigozanthos bicolor* shoots was highest at a light intensity of 200 µmol m^{-2} s^{-1} compared with 80 µmol m^{-2} s^{-1}. In *Alocasia amazonica*, plantlet growth was highest at a light intensity of 30 µmol m^{-2} s^{-1}. That particular study revealed that *A. amazonica* can perform well in low light intensity **(JO et al., 2008)**. Although light is the energy source for plant growth, excess light may lead to photosynthetic efficiency depression or photo-

inhibition **(JO et al., 2008)**. A small difference of a few hours in the photoperiod can significantly affect the cost of *in vitro* propagation in terms of energy consumption. It is therefore necessary to determine the minimum photoperiod required for satisfactory plant growth *in vitro* **(MORINI et al., 1991)**.

2.5 MICROPROPAGATION OF *DIERAMA* SPECIES

2.5.1 Explant sources

Various explants have been used in the family Iridaceae to achieve successful micropropagation. These include anthers, bulbs, corms, flowers, hypocotyls, leaves, ovaries, plantlets, roots, shoots and twin scales. In the genus *Dierama* corms and hypocotyl explants have been used **(PAGE and VAN STADEN, 1985, MADUBANYA et al., 2006)**.

2.5.2 *Dierama latifolium*

There are only two *Dierama* species for which a record of micropropagation has been published to date. **PAGE and VAN STADEN (1985)** established an *in vitro* propagation protocol for *Dierama latifolium.* In that study which involved the use of corms as explant sources, a higher number of explants formed shoots when grown on medium without PGRs, or supplemented with 0.5 mg L^{-1} NAA. Neither root proliferation nor shoot multiplication was significantly influenced by the addition of BA to the growth medium. Rooting of *in vitro*

propagated shoots was induced by subculturing excised shoots on PGR-free medium or medium supplemented with 0.5 or 1.0 mg L^{-1} NAA.

2.5.3 *Dierama luteoalbidum*

Hypocotyl explants of *D. luteoalbidum* formed multiple shoots on MS medium supplemented with 0.5 mg L^{-1} BA while an increase in BA beyond 0.5 mg L^{-1} and the addition of 1 mg L^{-1} NAA increased the incidence of callus **(MADUBANYA et al., 2006)**. Shoots cultured in a liquid-shake medium containing 0.5 mg L^{-1} BA produced meristemoids which, when transferred to solid medium, produced multiple shoots **(MADUBANYA et al., 2006)**.

2.6 FUTURE PROSPECTS

The demand of medicinal and ornamental plants has proven to have a negative impact on the wild populations. Conventional propagation methods will go a long way in solving this problem. While *in vitro* propagation seems to be a solution for plant species decimation, successful micropropagation protocols need to be developed for many other species in the Iridaceae family. Effects of environmental factors such as light and temperature *in vitro* have not been reported in micropropagation of *Dierama* species. These factors need to be studied as they may bring about further optimization of the existing micropropagation protocols.

CHAPTER THREE:
SHOOT INITIATION AND PROLIFERATION

3.1 INTRODUCTION

Micropropagation is the science of growing plant cells, tissues or organs obtained from a plant on an artificial medium **(GEORGE et al., 2008)**. Before micropropagation commences, careful attention needs to be given to the stock plants from which explants are selected and obtained. Stock plants and hence explants must be free from microbial contaminants and any symptoms of diseases. For explant decontamination, various surface sterilants such as 70% v/v ethanol, sodium hypochlorite and mercuric chloride are used **(PIERIK, 1987)**.

Following successful decontamination, multiplication of plantlets can be initiated from newly-derived axillary or adventitious shoots **(GEORGE, 1993)**. In any given micropropagation procedure, the choice of explants from which shoots are initiated is critical. In the Iridaceae, the most common choice of explants has been from corms and inflorescences **(ZIV and LILIEN-KIPNIS, 2000)**. Inflorescence explants were used to induce callus from which shoots, roots and later corms were produced **(ZIV et al., 1970)**. Hypocotyls were used for successful micropropagation of *Dierama luteoalbidum* **(MADUBANYA, 2004)** and corm explants of *Dierama latifolium* were used for plant regeneration **(PAGE and VAN STADEN, 1985)**. The present study gives an account on the use of corms, hypocotyls and ovaries as explant sources in the *in vitro* propagation of *Dierama erectum*. To further optimize this protocol, this Chapter

also deals with the investigation of photoperiod and light intensity effects on *D. erectum* shoot multiplication.

3.2 MATERIALS AND METHODS

3.2.1 CULTURE ESTABLISHMENT FROM SEEDLING SECTIONS

3.2.1.1 Seed decontamination and germination

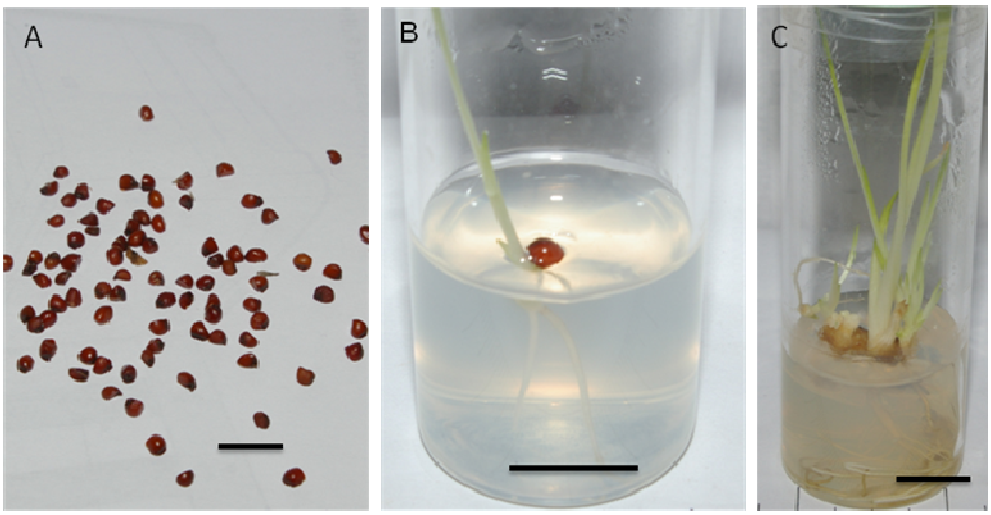


Figure 3.1: *In vitro* propagation of *Dierama erectum.* Seeds (**A**). *In vitro* seed germination (**B**). Shoot multiplication on MS medium supplemented with 1.0 µM BA (**C**). Bar = 1.0 cm.

Seeds (**Figure 3.1A**) were collected from Mt. Gilboa, KwaZulu-Natal (S 29° 15.873", E 30° 29.743 " ± 5 m) in April, 2008 and were pre-treated with 70% v/v ethanol for 1 min. Seeds were surface decontaminated for 20 min in either 0.2% mercuric chloride ($HgCl_2$), 2.5% w/v sodium hypochlorite (NaOCl), 3.5% v/v

NaOCl, 2.5% v/v NaOCl combined with 1% benomyl (Benlate®), or 1% w/v copper oxychloride (Virikop®). Polyoxyethylene sorbitan monolaurate (Tween® 20) was used as a surfactant in the sterilants. Seeds were rinsed three times with sterile distilled water and inoculated in individual test tubes containing 10 ml of 1/10th strength **MURASHIGE and SKOOG (1962)** medium (MS) without sucrose or plant growth regulators. There were 100 seeds per treatment and these were allowed to germinate (**Figure 3.1B**) at 25 ℃ for 21 days in 16 h light.

3.2.1.2 Shoot initiation from seedling sections

Seedlings were used for this experiment once they were about 10 cm long. Seedlings were divided into three sections: the root, hypocotyl and leaf. They were cut into about 5 mm lengths and inoculated on 10 ml (in culture tubes, 100 mm × 25 mm, 40 ml volume) MS medium supplemented with 30 g L^{-1} sucrose, 0.1 g L^{-1} myo-inositol and various concentrations (0.0, 2.0, 5.0, 10.0 µM) of N^6-benzyladenine (BA). There were 20 replicates per treatment. After 8 weeks, the number of adventitious shoots produced by each explant type from each treatment was recorded.

3.2.1.3 Effects of different auxin and cytokinin combinations on shoot multiplication

Following seed decontamination and germination, hypocotyl explants were excised from seedlings (as they were found to be the only regenerative

explants). Hypocotyls were cut into about 5 mm lengths and inoculated on 10 ml (culture tubes, 100 mm × 25 mm, 40 ml volume) MS medium supplemented with 30 g L^{-1} sucrose, 0.1 g L^{-1} myo-inositol and various concentrations of the cytokinins; N^6-benzyladenine (BA), *meta*-topolin (*m*T), kinetin (KIN), and zeatin (Z) with or without the auxin 1-naphthaleneacetic acid (NAA). The cytokinin concentrations used were 0.0, 0.5, 1.0, 2.0, 5.0, and 10 µM while NAA concentrations were 0.0, 0.5, 1.0 and 2.0 µM. It must be noted that the Z used in this study was a racemic mixture of *cis*- and *trans*-zeatin. These experiments were arranged in a 5 × 4 factorial design. The pH was adjusted to 5.8 with 1 N KOH or 1 N HCl. The medium was solidified with 0.8% w/v agar and autoclaved at 121℃ and 103 kPa for 20 min. Cultures were allo wed to grow under 16 h light (30 µmol m^{-2} s^{-1}) at 25 ℃. Each treatment had 15 replicates. After 8 weeks in culture, mean number of adventitious shoots per explant, percentage of explants producing shoots, percentage of explants producing roots and percentage of explants producing callus were recorded.

3.2.1.4 Effects of photoperiod and light intensity on shoot multiplication

After bulking up sufficient plant material on MS medium supplemented with 1.0 µM BA (**Figure 3.1C**), intact individual shoots of 1 cm length were sub-cultured in culture jars containing 30 ml MS medium supplemented with 1.0 µM BA. Eight different light conditions were tested in this experiment. Cultures were incubated in either 16 h light or 24 h light at 25 ℃ under the following light intensities: 10, 30, 70 and 100 µmol m^{-2} s^{-1}. Each treatment in this experiment

consisted of 21 replicates. After 8 weeks in culture, number of adventitious shoots per explant, shoot length and shoot fresh weight were recorded.

3.2.1.5 Effects of photoperiod and light intensity on chlorophyll and total carotenoid content

To determine chlorophyll and total carotenoid content, samples obtained from the photoperiod and light intensity experiments were weighed and extracted separately in 100% acetone (50 ml g^{-1} fresh weight). They were crushed using a mortar and pestle and filtered through Whatman No. 1 filter paper under vacuum. The filtrate was centrifuged using a Hettich Universal centrifuge (Tiiv Bayer, Germany) at 2500 rpm (revolutions per min) for 10 min. The supernatant was separated and the absorbance was read at 470, 645 and 662 nm using a spectrophotometer (Cary 50 UV-Visible spectrophotometer, Varian, Australia). There were five replicates from each treatment. The amount of chlorophyll *a*, *b* and total carotenoids were determined using the following formulae provided by **DERE et al.(1998)**:

$C_a = 11.75\ A_{662} – 2.350\ A_{645}$

$C_b = 18.61\ A_{645} – 3.960\ A_{662}$

$C_{x+c} = 1000\ A_{470} – 2.270\ C_a – 81.4\ C_b/227$

3.2.2 CULTURE ESTABLISHMENT FROM CORMS AND OVARIES

3.2.2.1 Culture initiation from corms

Plants were collected from Mt. Gilboa, KwaZulu-Natal in April, 2009. Corms were decontaminated using the procedure outlined by **PAGE and VAN STADEN (1985)**. Explants were washed under running tap water, immersed in 100% ethanol for 5 min, transferred to a 0.1% (w/v) mercuric chloride solution for 20 min, and then rinsed twice with sterile distilled water. The vertically cut sections (approximately 1 cm wide) were inoculated on full strength MS medium supplemented with either 1.0 µM BA, 5.0 µM KIN, 2.0 µM *m*T or PGR-free medium (these concentrations gave the best results in the previous experiments investigating the effects of PGRs on shoot multiplication). Cultures were allowed to grow under 16 h light (30 µmol m^{-2} s^{-1} light intensity) at 25 ℃ and each treatment had 15 replicates. After 8 weeks in culture, the number of explants producing shoots (%) and number of shoots per responding explants were recorded.

3.2.2.2 Culture initiation from ovaries

Unfertilised ovaries were obtained from plants growing in the wild (Mt. Gilboa KwaZulu-Natal) in March 2009. They were washed in tap water and pre-treated with 70% ethanol prior to surface decontamination with 3.5% sodium hypochlorite solution for 15 min. The explants were rinsed three times with sterile distilled water. Before culturing the ovaries, the tip of the distal part of the

pedicel was removed and individual ovaries were implanted with their cut ends inserted in full strength MS medium supplemented with either 1.0 µM BA, 5.0 µM KIN, 2.0 µM *m*T or hormone-free medium. Cultures were grown under 16 h light (30 µmol m^{-2} s^{-1} light intensity) at 25 ℃ and each treatment was re plicated 15 times.

3.3 DATA ANALYSIS

Where necessary, data were subjected to one-way analysis of variance (ANOVA) using SPSS version 10.0 or GenStat (11th edition). The significance level was determined at P = 0.05. Where there were significant differences, means for SPSS and GenStat outputs were separated using Duncan's Multiple Range Test (DMRT) and LSD (Least Significant Difference) respectively.

3.4 RESULTS AND DISCUSSION

3.4.1 Seed decontamination and germination

Results presented in **Table 3.1** show that successful decontamination was achieved when 0.2% $HgCl_2$ or 2.5% NaOCl + 1% Benlate® were used for 20 min after rinsing with 70% ethanol for 1 min. These treatments were associated with high seed germination percentages. Low decontamination percentage and thus low seed germination with 2.5% NaOCl and 1% Virikop® suggest that these concentrations were too low to kill the contaminants. Increasing NaOCl

concentration to 3.5% increased decontamination but seed germination percentage was still low, implying that 3.5% NaOCl may have been too high and toxic to the seeds. This suggests that without a proper decontamination procedure, seed germination is suppressed. As heavy metal ions such as mercuric chloride are highly toxic and their use is not preferred **(MADUBANYA, 2004)**, the best surface decontamination agents for *D. erectum* seeds are 2.5% NaOCl combined with 1% Benlate®.

3.4.2 Shoot initiation from seedling sections

Hypocotyl explants used in this study showed shoot response between the fourth and sixth week of culture in all MS medium types, while leaf and root explants showed no response at all. In this preliminary investigation, hypocotyl explants gave a maximum shoot response at 2.0 and 5.0 µM BA (**Table 3.2**). **ASCOUGH et al. (2009b)** stated that the observed hypocotyl response might be because in most monocotyledonous plants, of which *D. erectum* is one, the hypocotyl section has greater regenerative capacity than other seedling sections and produces shoots when excised.

Table 3.1: Effect of surface-sterilizing agents on decontamination and germination of *D. erectum* seeds

Treatment	Duration (min)	Decontamination (%)	Seed germination (%)
2.5% NaOCl	20	40	47
3.5% NaOCl	20	72	51
0.2% $HgCl_2$	20	88	86
2.5% NaOCl + 1% Benlate®	10 + 10	86	79
1% Virikop®	20	56	41

Table 3.2: The response of different seedling sections to BA

BA concentration (µM)	No. of adventitious shoots produced per explant		
	Root	Hypocotyl	Leaf
0.0	0	1.5 ± 0.48^{b}	0
2.0	0	3.3 ± 1.12^{a}	0
5.0	0	3.0 ± 1.53^{a}	0
10.0	0	1.7 ± 0.64^{b}	0

Values with different letter(s) indicate significant differences between treatments ($P = 0.05$) based on DMRT (n = 20).

3.4.3 Effects of different auxin and cytokinin combinations on shoot multiplication

The effects of different combinations of BA and NAA on shoot multiplication are presented in **Figure 3.2**. An increase of BA concentration alone to 1.0 µM in the medium, increased shoot multiplication (2.40 ± 0.40 shoots per explant) which then declined with concentrations beyond this level. In general, addition of 0.5 µM NAA to BA, did not yield significant shoot multiplication. However, by increasing the NAA concentration to 1.0 µM, shoot multiplication increased significantly with increasing concentration of BA but dropped with addition of 10.0 µM BA. The highest number of shoots (3.20 ± 0.22 shoots per explant) with 100% explants showing shoot response, 13% explants showing root response and 27% of explants showing callus formation were obtained from medium supplemented with 1.0 µM NAA + 2.0 µM BA (**Figure 3.2 A-D**). A combination of 2.0 µM NAA at all BA concentrations resulted in a decrease in shoot multiplication. Shoot multiplication inhibition by the auxin may be as a result of down-regulation of cytokinin biosynthesis and promotion of cytokinin metabolic inactivation **(KAMÍNEK et al., 1997)**.

BA has been used extensively for micropropagation of *Dierama* species and closely related genera in the Iridaceae. Successful micropropagation of *Dierama* was first reported by **PAGE and VAN STADEN (1985)**, where 0.5 – 1.0 mg L^{-1} BA (2.2 – 4.4 µM) were used for the induction of multiple shoots in *D. latifolium*. Later, **MADUBANYA et al. (2006)** achieved successful

micropropagation of *D. luteoalbidum* by supplementing the MS medium with 0.5 mg L^{-1} BA (2.2 µM).

Other Iridaceae species where BA was applied include: *Iris hollandica* **(HUSSEY, 1976)**, *Gladiolus* species **(DANTU and BHOJWANI, 1995)**, *Babiana* species **(McALISTER, et al., 1998)**, and *Watsonia* species **(ASCOUGH et al., 2007)**. The high biological activity of BA at low concentrations (such as 1.0 µM in the present study, **Figure 3.2A**) and its relatively lower cost **(WERBROUCK et al., 1996)** may be the reasons for its widespread use in micropropagation systems. The increased biological activity of BA over KIN has been attributed to its form of ring substitution - replacement of the furfuryl group by a benzyl group **(SKOOG and ARMSTRONG, 1970)**.

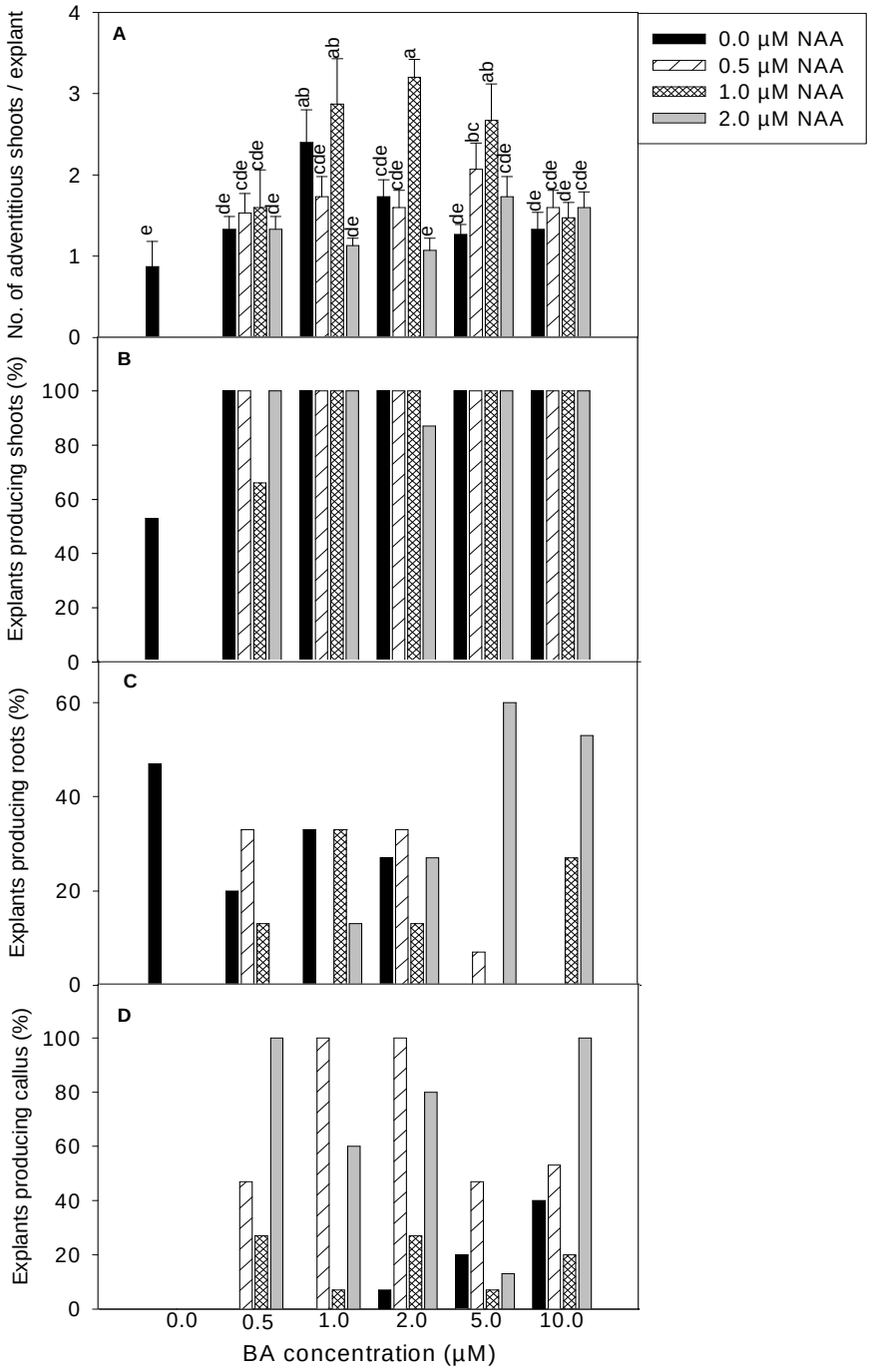


Figure 3.2: Effect of BA and NAA on shoot multiplication (**A**), explants producing shoots (**B**), explants producing roots (**C**) and explants forming callus (**D**) in *D. erectum*. Bars with different letters indicate significant differences between treatments (P = 0.05, n = 15).

Figure 3.3 shows the effects of different combinations of *m*T and NAA on shoot multiplication in *D. erectum*. Increasing concentrations of *m*T without NAA marginally increased shoot multiplication though not significantly different from the control whereas, addition of 0.5 µM NAA to 1.0 µM *m*T significantly increased shoot multiplication. There was no particular trend observed with the addition of 1.0 µM NAA to all *m*T concentrations. Increasing the concentration of *m*T in the presence of 2.0 µM NAA in the growth medium generally resulted in increased shoot multiplication. The highest shoot production (3.09 ± 0.99 shoots per explant) with 100% explants producing shoots was obtained with the addition of 2.0 µM NAA and 2.0 µM *m*T to the growth medium.

In as much as can be ascertained, there is no report on the use of topolins in micropropagation of the Iridaceae. Their successful use however, has been reported on micropropagation of various species, most notably, *Aloe ferox*, *Aloe polyphylla* **(BAIRU et al., 2007)**, *Harpagophytum procumbens* **(BAIRU et al., 2009)**, *Spathiphyllum floribundum* **(WERBROUCK et al., 1996)**, *Petunia hybrida* and *Rosa hybrida* **(BOGAERT et al., 2006)**. Most of these reports also indicated the superiority of topolins both in shoot multiplication and controlling *in vitro* abnormalities such as early senescence in wheat **(PALAVAN-ÜNSAL et al., 2002)**. The position of the hydroxyl group in the side chain has a significant effect on the biological activity of a cytokinin. The hydroxylation of the phenyl ring in the *meta* position (in this case *m*T) contributes to the increased cytokinin activity **(KAMÍNEK et al., 1987)**. In the case of micropropagation of *D. erectum* however, *m*T was not found to be superior to BA, KIN or Z (**Table 3.3**).

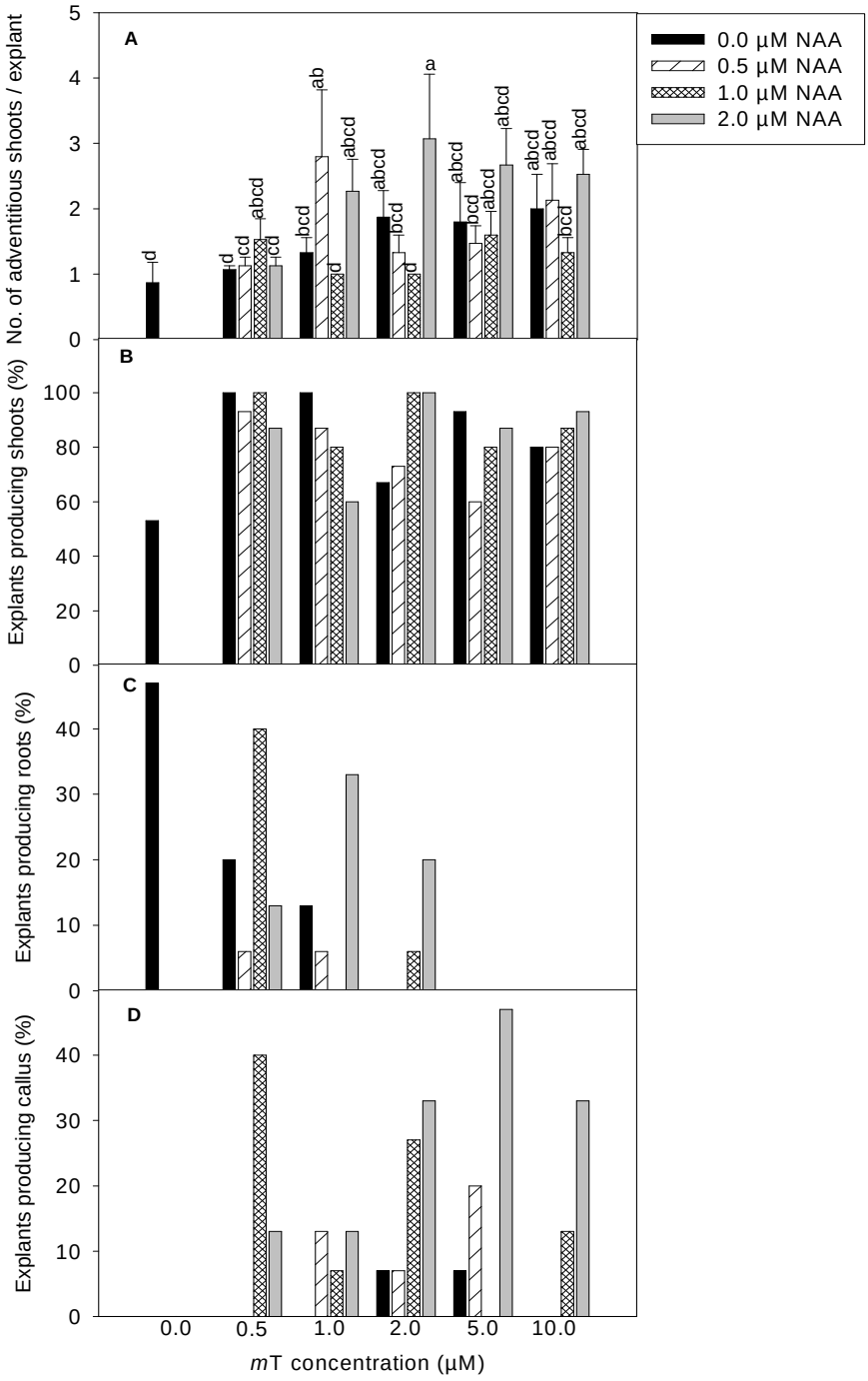


Figure 3.3: Effect of *m*T and NAA on shoot multiplication (**A**), explants producing shoots (**B**), explants producing roots (**C**) and explants forming callus (**D**) in *D. erectum*. Bars with different letters indicate significant differences between treatments (P = 0.05, n = 15).

The effects of KIN and NAA on shoot multiplication are presented in **Figure 3.4**. Increasing concentrations of KIN alone in the growth medium resulted in an increase in shoot multiplication and this was highest at 5.0 µM but dropped when 10.0 µM KIN was added to the growth medium. Addition of 0.5 µM NAA to 2.0 µM KIN resulted in the highest shoot multiplication (3.67 ± 0.81 shoots per explant). Interestingly, this combination resulted in 100% of the explants showing a shoot response (**Figure 3.4B**) and no callus formation (**Figure 3.4D**). Addition of 1.0 µM NAA to the growth medium resulted in increasing shoot multiplication with increasing concentrations of KIN but this was only up to 5.0 µM KIN where a higher shoot production was observed. Better shoot production was again found when 2.0 µM NAA was combined with 5.0 µM KIN.

As can be seen in **Figure 3.4A**, KIN application alone at concentrations below 5.0 µM did not produce a significantly high number of shoots. This observation agrees with the statement that KIN has relatively low biological activity in certain bio-assays **(BOGAERT et al., 2006)**. At lower concentrations, KIN has lower activity when compared to other cytokinins such as BA **(BENTLY-MOWAT and REID, 1968; TAKAYAMA and MISAWA, 1982)** and therefore may require a higher concentration to achieve significant shoot multiplication rates. However, for *in vitro* propagation of *D. erectum*, KIN was not found to be weak at lower concentrations (2.0 µM) especially when combined with 0.5 µM NAA (**Table 3.3**), rather this combination resulted in the second best shoot multiplication.

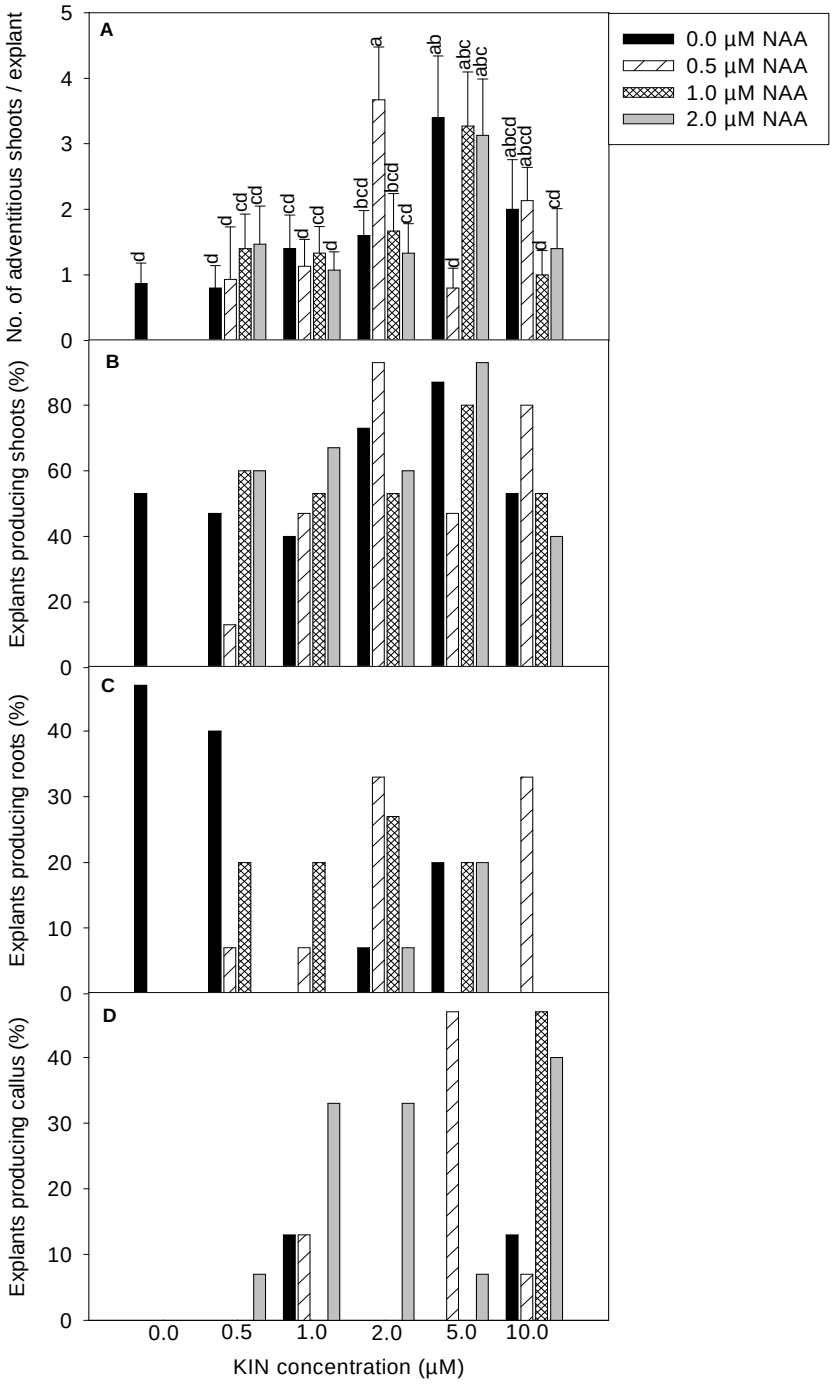


Figure 3.4: Effect of KIN and NAA on shoot multiplication (**A**), explants producing shoots (**B**), explants producing roots (**C**) and explants forming callus (**D**) in *D. erectum*. Bars with different letters indicate significant differences between treatments (P = 0.05, n = 15).

Figure 3.5 presents the effects of Z and NAA on shoot multiplication of *D. erectum*. It can be seen that increasing the concentration of Z resulted in increased shoot multiplication and the optimum concentration was 1.0 µM Z, giving a shoot multiplication of 4.20 ± 0.51 shoots per explant. Adding 0.5 µM NAA to the growth medium also increased shoot multiplication with increasing Z concentrations, reaching the highest at 2.0 µM Z. However, there was no significant difference between all Z concentrations combined with 0.5 µM NAA. Addition of 1.0 µM NAA to all concentrations of Z increased shoot multiplication and this was much higher when combined with 1.0 µM Z. Increasing the concentration of auxin to 2.0 µM generally lowered shoot multiplication. The highest shoot multiplication rate was obtained at a concentration of 1.0 µM Z without NAA. There were no significant differences observed between the treatments except the control and the medium containing 10 µM Z plus 2.0 µM NAA. It is well documented that higher concentrations of auxins (in this case 2.0 µM NAA) inhibit shoot multiplication **(BAIRU et al., 2008)**.

The *trans* isomer of Z has been reported as the most active form of the naturally occurring cytokinins **(MARTIN et al., 1999)**. The *trans*-hydroxylation of the N^6 isoprenoid side chain of Z and the presence of a double bond at the 2, 3-position of the side chain **(MOK et al., 1978)**, gives it high cytokinin activity **(WHITTY and HALL, 1974)**. This may be a reason for its ability to induce a high number of shoots in a wide range of concentrations (from 0.5 to 10.0 µM as observed in this study). Z has been used in micropropagation of some Iridaceae species for; induction of primary and secondary embryogenesis in *Gladiolus* **(REMOTTI, 1995)**, increasing the reproductive capacity of *Crocus sativus*

(PLESSNER et al., 1990) and plant regeneration from *Gladiolus garnierrii* **(REMOTTI and LÖFFLER, 1995)**. These authors reported enhanced shoot multiplication rates with the use of Z.

As can be seen from **Table 3.3**, the highest shoot multiplication (4.2 shoot per explant) was achieved when Z (1.0 µM) was used in the growth medium. Although this value was not significantly different from others (in bold), these results suggest that for better shoot multiplication in *D. erectum* Z can be used, given that the cost of Z is not a limiting factor.

It is evident from this study that the different cytokinins used yielded different shoot numbers at different concentrations with or without NAA. These observed differences may be due to the differences in cytokinin uptake and metabolism in plant cells **(VAN STADEN and CROUCH, 1996)**. In addition, cytokinin efficacy may be affected by different affinities of cytokinin receptors involved in shoot induction **(KAMÍNEK et al., 1997)**.

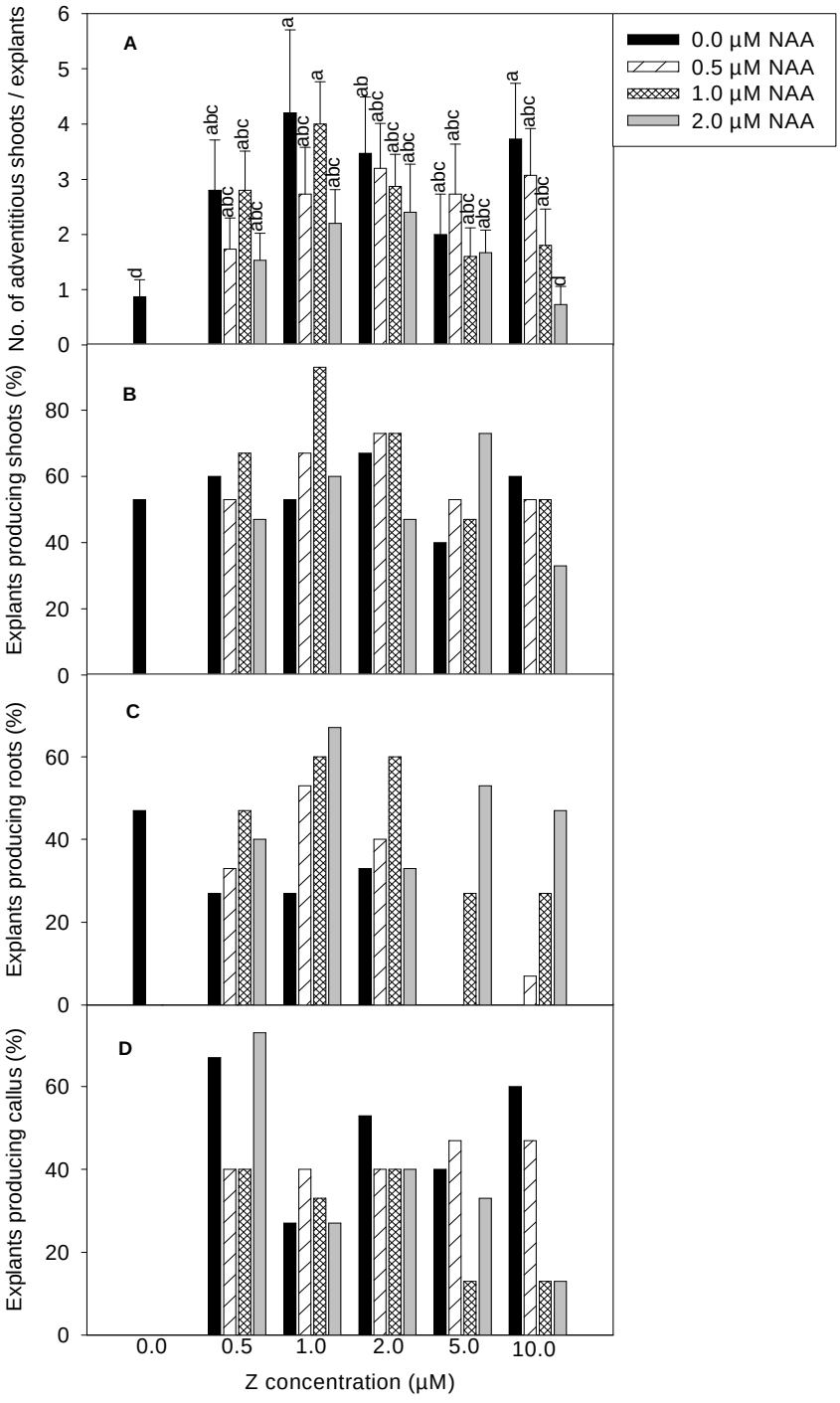


Figure 3.5: Effect of Z and NAA on shoot multiplication (**A**), explants producing shoots (**B**), explants producing roots (**C**) and explants forming callus (**D**) in *D. erectum*. Bars with different letters indicate significant differences between treatments (P = 0.05, n = 15).

Table 3.3: Effects of different combinations of cytokinins and NAA on shoot multiplication of *D. erectum*

Cytokinins (µM)				NAA (µM)			
BA	*m*T	KIN	Z	0.0	0.5	1.0	2.0
0.0	-	-	-	0.867			
0.5	-	-	-	0.133	1.533	1.600	1.333
1.0	-	-	-	2.400	1.733	2.867	1.133
2.0	-	-	-	1.733	1.600	**3.200**	1.067
5.0	-	-	-	1.267	2.067	2.667	1.733
10.0	-	-	-	1.333	1.600	1.467	1.600
	0.5	-	-	1.067	1.133	1.533	1.133
	1.0	-	-	1.333	2.800	1.000	2.267
	2.0	-	-	1.867	1.333	1.000	**3.067**
	5.0	-	-	1.800	1.467	1.600	2.667
	10.0	-	-	2.000	2.133	1.333	2.533
		0.5	-	0.800	0.933	1.400	1.467
		1.0	-	1.400	1.133	1.333	0.400
		2.0	-	0.800	**3.667**	1.667	1.408
		5.0	-	3.206	1.400	3.267	3.133
		10.0	-	2.000	2.133	1.000	1.400
			0.5	2.800	1.733	2.800	1.533
			1.0	**4.200**	2.733	4.000	2.200
			2.0	3.467	3.200	2.867	2.400
			5.0	2.000	2.733	1.600	1.667
			10.0	3.733	3.067	1.800	0.733

F. Probability < 0.001

LSD = 1.596

In this table, all the different combinations of cytokinins and NAA were analysed together for their statistical significance using GenStat 11th edition. Values in bold indicate the highest shoot multiplication obtained per cytokinin.

3.4.4 Effect of photoperiod and light intensity on shoot multiplication

Photoperiod and light intensity had significant effects on *in vitro* shoot multiplication of *D. erectum.* Maximum shoot production (12.73 ± 1.03 shoots per explant) was achieved when plants were grown in 16 h light at a light intensity of 100 µmol m^{-2} s^{-1} (**Figure 3.6** and **3.7A**). It is important to highlight that this multiplication rate was approximately four times higher than what was previously achieved in the present study with experiments investigating the effects of different cytokinins on shoot multiplication (refer to Table 3.3). This is in agreement with previous reports showing that control of light intensity and photoperiod can be used in tissue culture to improve plant yields **(ECONOMOU and READ, 1987)**.

It is also interesting to note that under similar light conditions (16 h light at 100 µmol m^{-2} s^{-1}), the highest number of shoots growing beyond 2 cm (4.13 ± 0.82 shoots) in height were obtained (**Figure 3.7B**). The highest total fresh weight of 0.67 ± 0.10 g per explant (**Figure 3.7C**) was also obtained under the 16 h light and 100 µmol m^{-2} s^{-1} treatment. Dark periods (8 h darkness) may favour synthesis or accumulation of substances which in turn favour growth in 16 h light periods **(ECONOMOU and READ, 1986)**. Moreover, increasing light intensity to 100 µmol m^{-2} s^{-1} may increase photosynthetic activity, thereby increasing fresh weight in plantlets **(CABALEIRO and ECONOMOU, 1991)**. These results indicate that *D. erectum* requires 16 h light and high light intensity (100 µmol m^{-2} s^{-1}) for the best shoot multiplication and vegetative growth (**Figure 3.6D**). Photo-oxidation of endogenous IAA, due to over-exposure to

light **(ECONOMOU and READ, 1986)** may be an explanation for the reduced growth of *D. erectum* cultures grown under 24 h light periods. A further explanation for the observed decrease in shoot production, shoot length and total fresh weight may be the result of photo-inhibition caused by over-exposure to light **(DANIEL, 1997)**, or by a limitation of carbon dioxide supply under high light intensity **(WILLIAMS et al., 1992)**.

3.4.5 Effect of photoperiod and light intensity on chlorophyll and total carotenoid content

A number of parameters regarding the effect of photoperiod and light intensity on extractable chlorophyll and total carotenoids are presented in **Figure 3.8**. The highest amounts of chlorophyll *a* (347.8 ± 180.5 µg/ g fresh weight), chlorophyll *b* (454.2 ± 206.5 µg/ g fresh weight) and total carotenoid content (54008.2 ± 27542.5 µg/ g fresh weight) were obtained when plants were grown under 16 h light at 70 µmol m^{-2} s^{-1}. However, values obtained with this combination were not significantly different from other treatments.

These results suggest that the levels of extractable chlorophylls and carotenoid in *D. erectum* were not dependent on the photoperiod and light intensity under which plants were grown. The effect of light on the levels of chlorophylls and total carotenoid content *in vitro* is complex and depends on many biochemical and physiological factors. Further studies are required to achieve a better understanding of plant physiology involving light regimes *in vitro.*

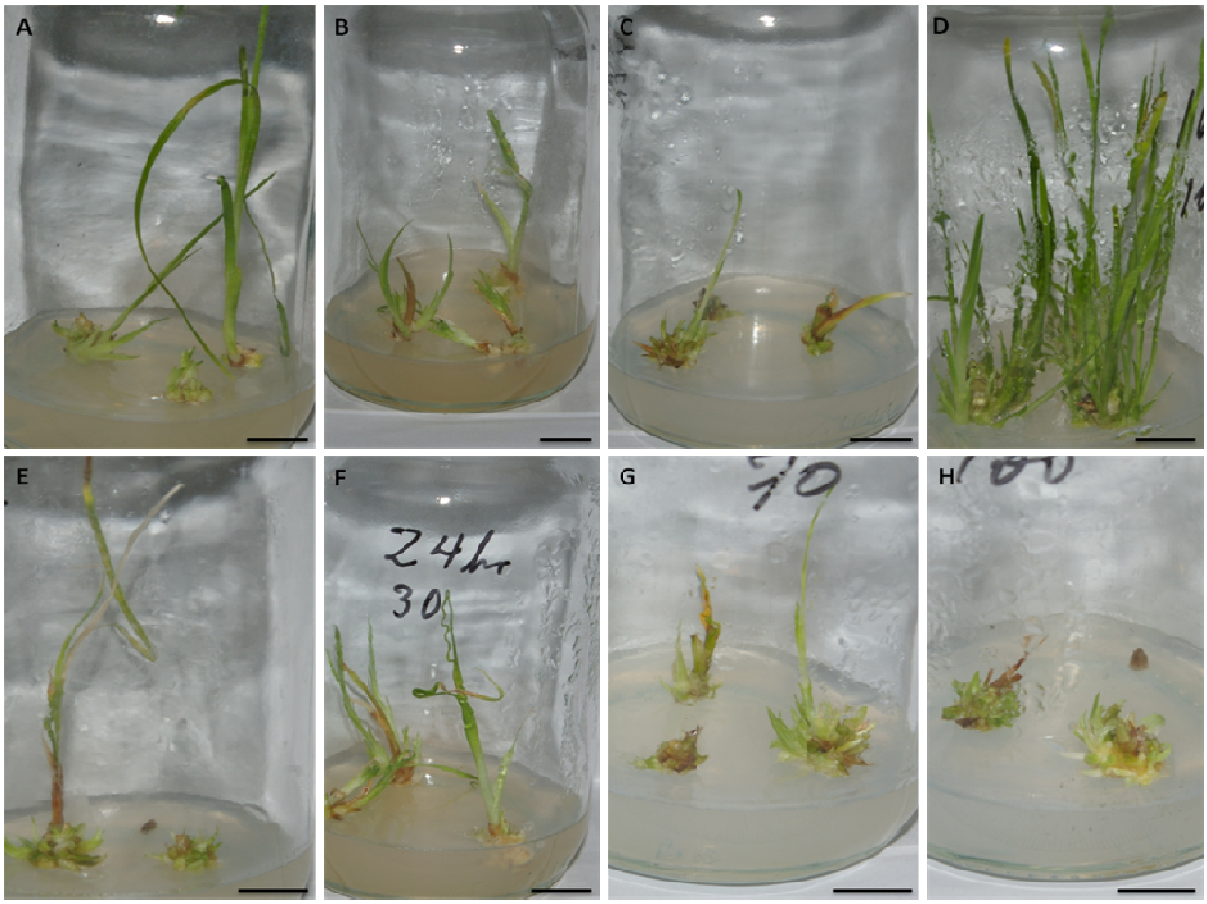


Figure 3.6: Effect of photoperiod and light intensity on shoot multiplication in *D. erectum*. **A**-**D** = 16 h light, **E**-**H** = 24 h light, **A** and **E** = 10 µmol m^{-2} s^{-1}, **B** and **F** = 30 µmol m^{-2} s^{-1}, **C** and **G** = 70 µmol m^{-2} s^{-1}, **D** and **H** = 100 µmol m^{-2} s^{-1}. Bar = 1.0 cm.

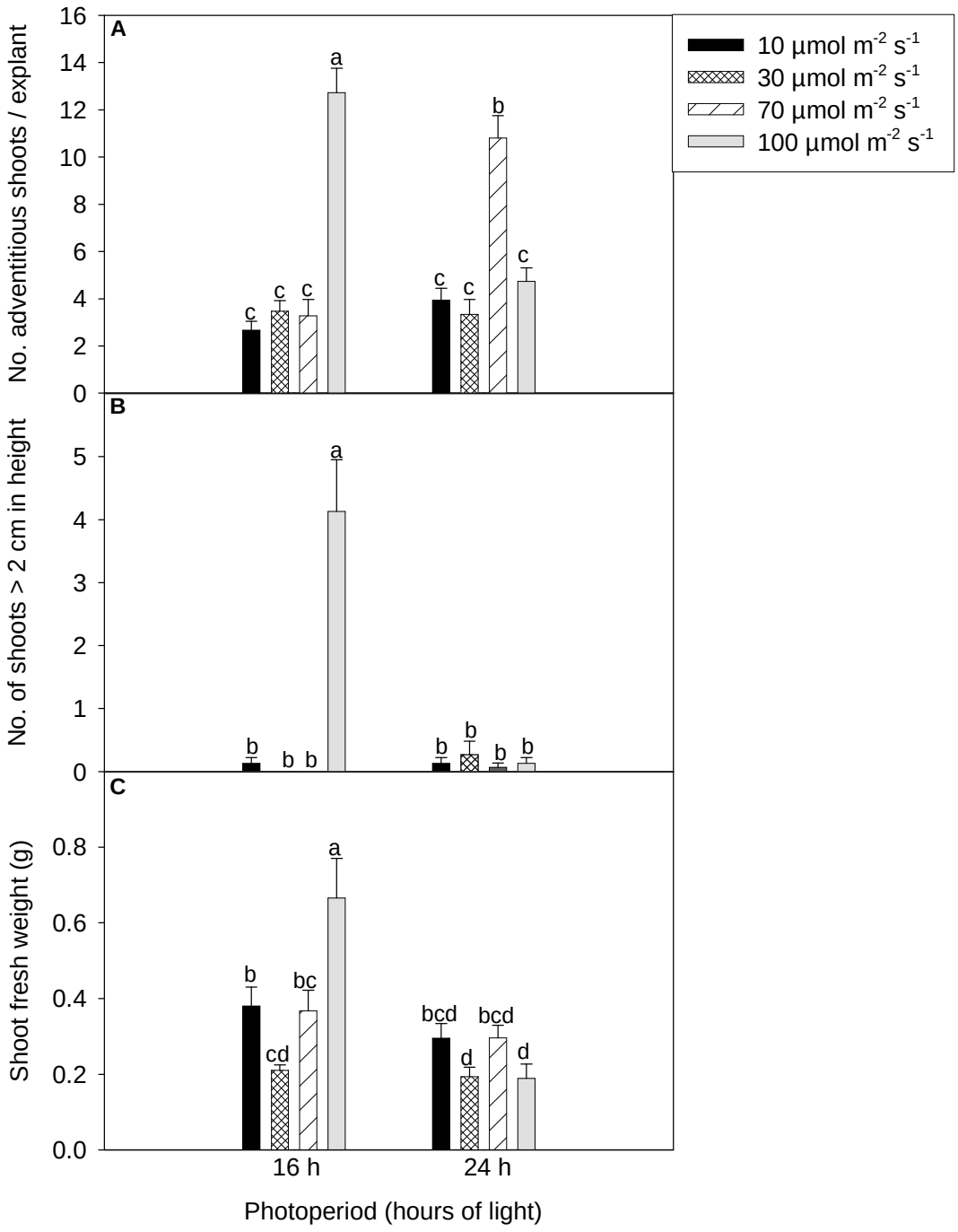


Figure 3.7: Effect of photoperiod and light intensity on the number of adventitious shoots (**A**), number of shoots > 2 cm (**B**) and total fresh weight (**C**) in *D. erectum*. Bars with different letters indicate significant differences between treatments (P = 0.05, n = 21).

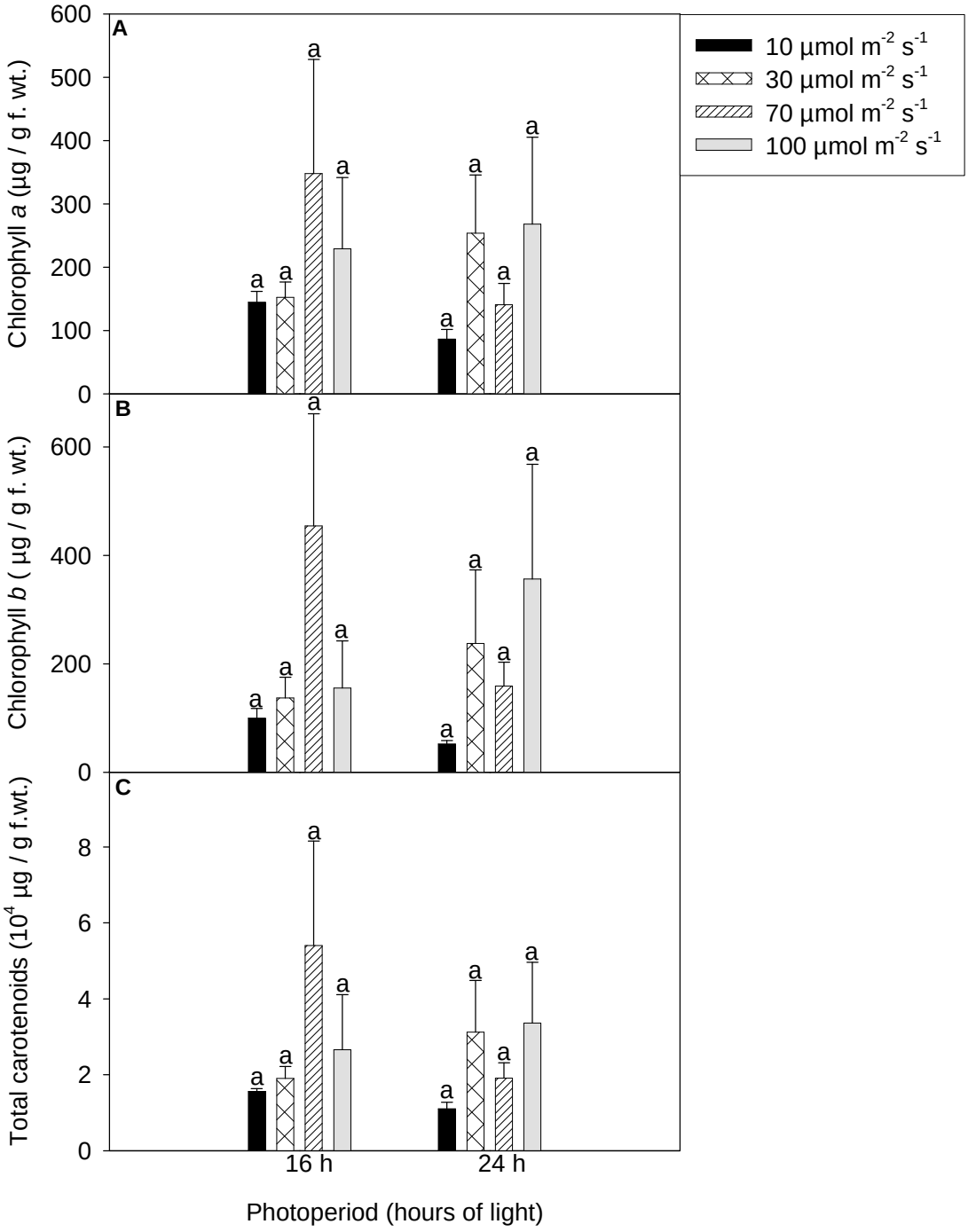


Figure 3.8: Effect of photoperiod and light intensity on chlorophyll *a* (**A**), chlorophyll *b* (**B**) and total carotenoids (**C**) in *D. erectum*. Bars with different letters indicate significant differences between treatments (P = 0.05, n = 21).

3.5 CULTURE INITIATION FROM CORMS AND OVARIES

3.5.1 Culture initiation from corms

Axillary shoots from which no adventitious shoots were produced, sprouted from corm segments cultured on MS medium (**Figure 3.9A-D**) and no callus formation was observed with all cytokinins applied. However, only a small percentage of corms responded to the given treatments. This could be due to corms being collected at the beginning of the winter season, rendering them less responsive. There were no significant differences between numbers of shoots produced in any of the treatments (**Table 3.4**).

3.5.2 Culture initiation from ovaries

Ovary explants did not yield any plantlets, but a small percentage of explants formed callus when cultured in basal medium supplemented with 1.0 µM BA, 5.0 µM KIN, 2.0 µM *m*T, and in the hormone-free medium. The callus turned brown after four weeks of incubation (**Figure 3.9 E-H**). **HUSSEY (1975)** reported that plantlets were induced from ovary wall tissues of some species in Liliaceae, while Iridaceae species did not produce plantlets from ovaries.

Table 3.4: The response of *D. erectum* corm explants to different cytokinins

Treatments	Explants producing shoots (%)	No. of shoots/responding explants
Control	20	2.00 ± 0.82[a]
1.0 µM BA	15	1.00 ± 0.00[a]
2.0 µM *m*T	10	1.00 ± 0.00[a]
5.0 µM KIN	15	1.33 ± 0.33[a]

Mean values followed by the same letter(s) in a column are not significantly different (*P* = 0.05) based on DMRT (n = 20).

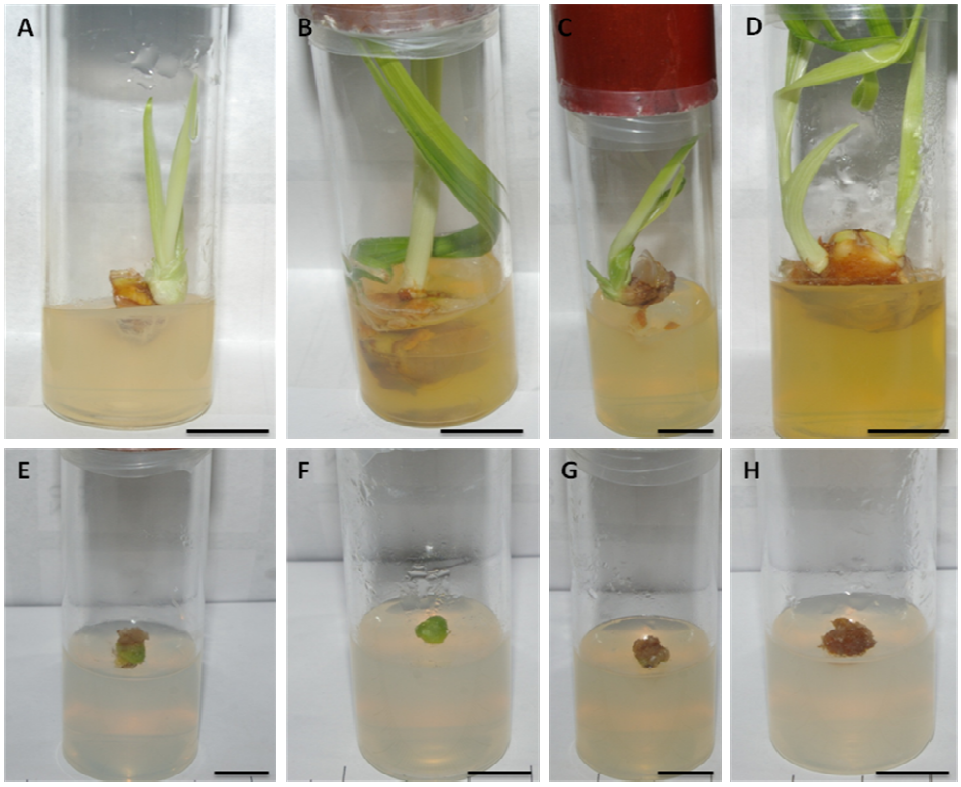


Figure 3.9: Culture initiation from *D. erectum* corms (**A-D**) and ovaries (**E-H**). **A** and **E** = control, **B** and **F** = 1.0 µM BA, **C** and **G** = 2.0 µM *m*T, **D** and **H** = 5.0 µM KIN. Bar = 1.0 cm.

3.6 SUMMARY

- Decontamination of seeds was effective when 0.2% $HgCl_2$ or 2.5% NaOCl combined with 1% Benlate were used.
- Hypocotyl and corm explants showed shoot regeneration potential while ovaries, leaves and roots did not give any shoot response.
- The highest shoot production with BA treatments was achieved when 2.0 µM BA was combined with 1.0 µM NAA.
- With *m*T treatments, maximum shoot production was obtained in the MS medium supplemented with 2.0 µM *m*T + 2.0 µM NAA.
- KIN yielded maximum shoots when 2.0 µM KIN was combined with 0.5 µM NAA in the growth medium.
- The highest shoot production with Z treatments was obtained in the medium supplemented with 1.0 µM Z without NAA.
- Shoot production was enhanced when *D. erectum* shoots were grown in 16 h light at a light intensity of 100 µmol m^{-2} s^{-1}.
- Extractable chlorophylls (*a* and *b*) and total carotenoid content were highest in plants grown in 16 h light at a light intensity of 70 µmol m^{-2} s^{-1}.

CHAPTER FOUR:
CORM INDUCTION, ROOTING AND ACCLIMATIZATION

4.1 INTRODUCTION

Micropropagation techniques offer great possibilities for mass propagation of many medicinal and horticulturally important plant species, however there will be no benefits from these techniques if *in vitro* regenerated plants are unable to survive stressful *ex vitro* environments **(ASCOUGH et al., 2008b)**. To avoid additional losses in micropropagation, due to poor rooting systems, rooting and storage organ induction (in this case corms) are necessary to facilitate plant survival during acclimatization **(DE KLERK et al., 1999)**. Benefits of *in vitro* corm formation include: (1) the possibility to reduce time to flowering because many ornamental geophytes need to go through several growth seasons before production of marketable inflorescences, (2) avoiding the cost of undertaking *in vitro* rooting and (3) increased survival rates **(ASCOUGH et al., 2008b)**.

Another available option for increasing plant survival rates *ex vitro* is an efficient rooting system. To achieve this, the auxins most commonly used are IAA, IBA and NAA. For *in vitro* rooting, different auxins may have different effects on a particular plant; hence this study involved testing the effectiveness of various auxins (IAA, IBA and NAA) for rooting of *Dierama erectum in vitro*.

Shoots derived *in vitro* may lack a functional cuticle and may have impaired stomatal functioning. This may result in high water loss and plantlets could be easily attacked by pathogens **(GEORGE, 1993)**. It is therefore essential to wean

plants in conditions of high relative humidity, shade and controlled temperatures after rooting **(PIERIK, 1987)**. The objective of the experiments presented in this Chapter was to develop an efficient protocol for corm induction and rooting of *D. erectum*, thus facilitating plant survival *ex vitro*.

4.2 MATERIALS AND METHODS

4.2.1 Effects of various concentrations of ancymidol, activated charcoal and sucrose on corm formation

Shoots obtained from the multiplication medium (MS + 1.0 µM BA) were cultured on full strength MS medium supplemented with activated charcoal (0.0, 0.1, 0.5 and 0.6% w/v) and sucrose (0, 1, 2, 3, 4 and 5% w/v) in a 4 × 6 factorial experiment. The ancymidol concentrations tested were: 0.0, 0.5, 1.0, 4.0, 8.0, and 16 µM. Each treatment consisted of 15 replicates. Cultures were grown in 16 h light (30 µmol m^{-2} s^{-1} light intensity) at 25 ℃. After 3 months in culture, shoot length, root length, leaf area and total fresh weight were recorded. The leaf area was measured using the area meter (LI-3100, LI-COR, inc. Nebraska, USA).

4.2.2 Effects of auxins on rooting

Following successful shoot multiplication on MS medium supplemented with 1.0 µM BA, newly formed plantlets were trimmed into about 1 cm lengths and inoculated on media containing IAA, IBA and NAA at concentrations of: 0.0, 0.5

and 1.0 µM. Cultures were allowed to grow under 16 h light (30 µmol m^{-2} s^{-1} light intensity) at 25 ℃. Each treatment had 21 re plicates. After 4 and 8 weeks in culture, number of roots and root length per treatment were recorded.

4.2.3 Acclimatization of plantlets to an *ex vitro* environment

Plants obtained from the rooting media were potted separately (according to the types and concentrations of auxins) in trays containing a 1:1 ratio of soil: vermiculite. They were left in the mist house for 2 weeks after which they were transferred to the greenhouse where they were irrigated once a day. Survival percentages were recorded after 2 months. It was observed that plantlets formed corms *ex vitro* after this period, thus corm diameter was also recorded.

4.3 DATA ANALYSIS

Where necessary, data were subjected to one-way analysis of variance (ANOVA) using SPSS version 10.0. The significance level was determined at $P = 0.05$. Where there were significant differences, means were separated using DMRT.

4.4 RESULTS AND DISCUSSION

4.4.1 Effects of various concentrations of ancymidol, activated charcoal and sucrose on corm formation

4.4.1.1 Effects of ancymidol

The use of ancymidol did not result in corm formation. However, its addition to MS medium had a significant impact on the morphology of *D. erectum* (**Figure 4.1**). Increasing the concentration of ancymidol in the medium resulted in reduced shoot length. The significantly shortest shoots (3.13 ± 0.18 cm) were found in the medium supplemented with 16.0 µM while the longest (13.52 ± 0.84 cm) were found in the control treatment (**Figure 4.2A**). Similarly, the significantly highest root length (6.41 ± 0.36 cm) was obtained in the PGR-free medium while increased ancymidol concentrations generally resulted in stunted roots (**Figure 4.2B**).

Addition of 0.5 µM ancymidol in the growth medium resulted in maximum leaf area (2.62 ± 0.66 cm^2). The leaf area declined with increasing ancymidol concentrations beyond 0.5 µM (**Figure 4.2C**). The increase in the total fresh weight of *D. erectum* with the addition of ancymidol from 0.5 to 8.0 µM (0.77 ± 0.072 g being the maximum at 2.0 µM) was significantly different from the control and the 16.0 µM treatments (**Figure 4.2D**).

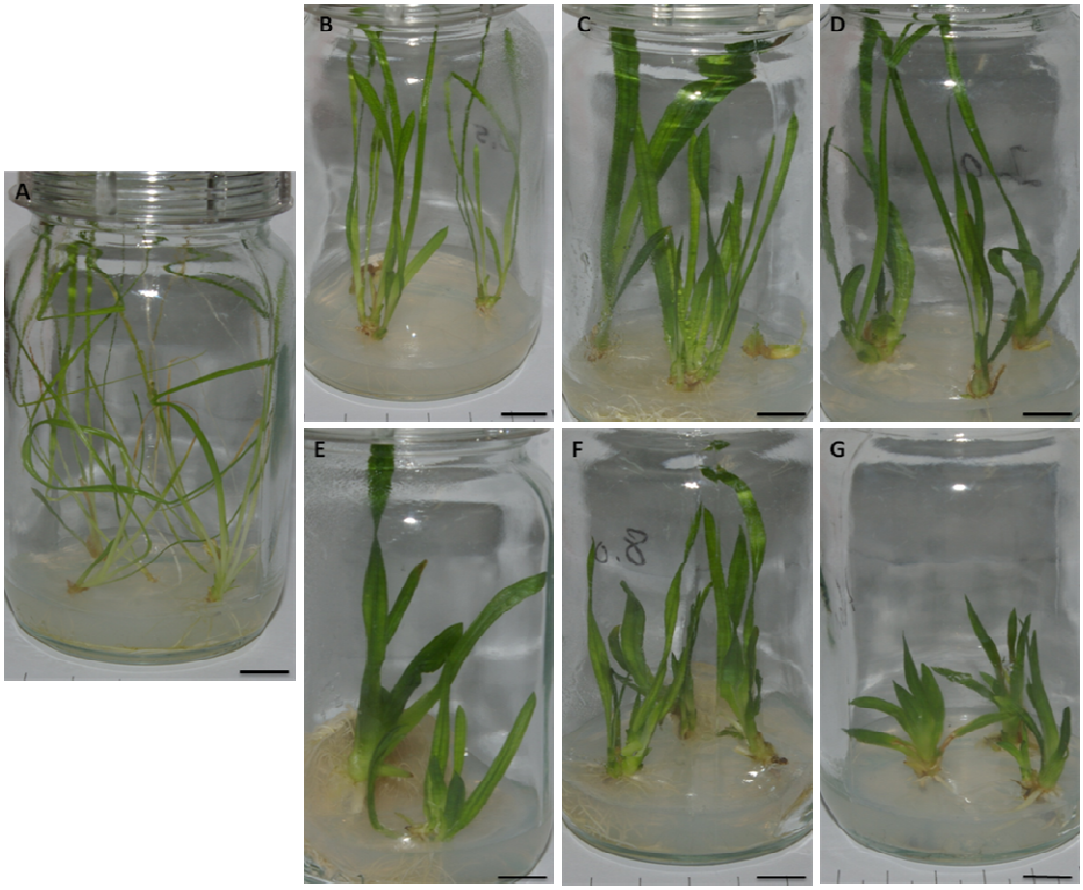


Figure 4.1: Effect of ancymidol concentrations on the morphology of *D. erectum.* **A** = control, **B** = 0.5 µM, **C** = 1.0 µM, **D** = 2.0 µM, **E** = 4.0 µM, **F** = 8.0 µM and **G** = 16.0 µM. Bar = 1.0 cm.

The use of growth retardants for storage organ induction is well known in micropropagation of ornamental geophytes such as *Dierama luteoalbidum* **(MADUBANYA et al., 2006)** and *Gladiolus* **(ZIV, 1989)**. Despite being successful for storage organ induction in *Gladiolus* **(ZIV, 1989)**, *Brodiaea*, *Philodendron* **(ZIV, 1992)** and *Crinum* **(SLABBERT et al., 1993)**, ancymidol did not promote corm formation in *D. erectum.* Similar trends were observed by **ASCOUGH et al. (2007)** where paclobutrazol (a growth retardant) did not induce corm formation in *Watsonia* species.

The observed decrease in shoot and root length (**Figure 4.2A** and **B**) may be due to the inhibition of gibberellin biosynthesis by ancymidol **(SHIVE and SISLER, 1976)**. Ancymidol inhibits elongation by blocking the steps involved in the *ent*-kaurine, *ent*-kaurenol and *ent*-kaurenal oxidation during gibberellin biosynthesis **(COOLBAUGH et al., 1982)**. The reduction in biomass with the use of ancymidol in micropropagation of *Philodendron* was attributed to inhibition of leaf growth **(ZIV and ARIEL, 1991)**. This was not the case with *D. erectum* where the reduction in shoot length, root length and leaf area due to addition of ancymidol generally did not cause a decrease in total fresh weight **(Figure 4.2D**). According to **LE GUEN-LE SAOS et al. (2002)**, the increase in fresh weight may be due to a better sucrose supply to the plants *in vitro*, triggered by the presence of ancymidol and resulting in more sucrose accumulation and its conversion to starch **(LORETI et al., 2001)**.

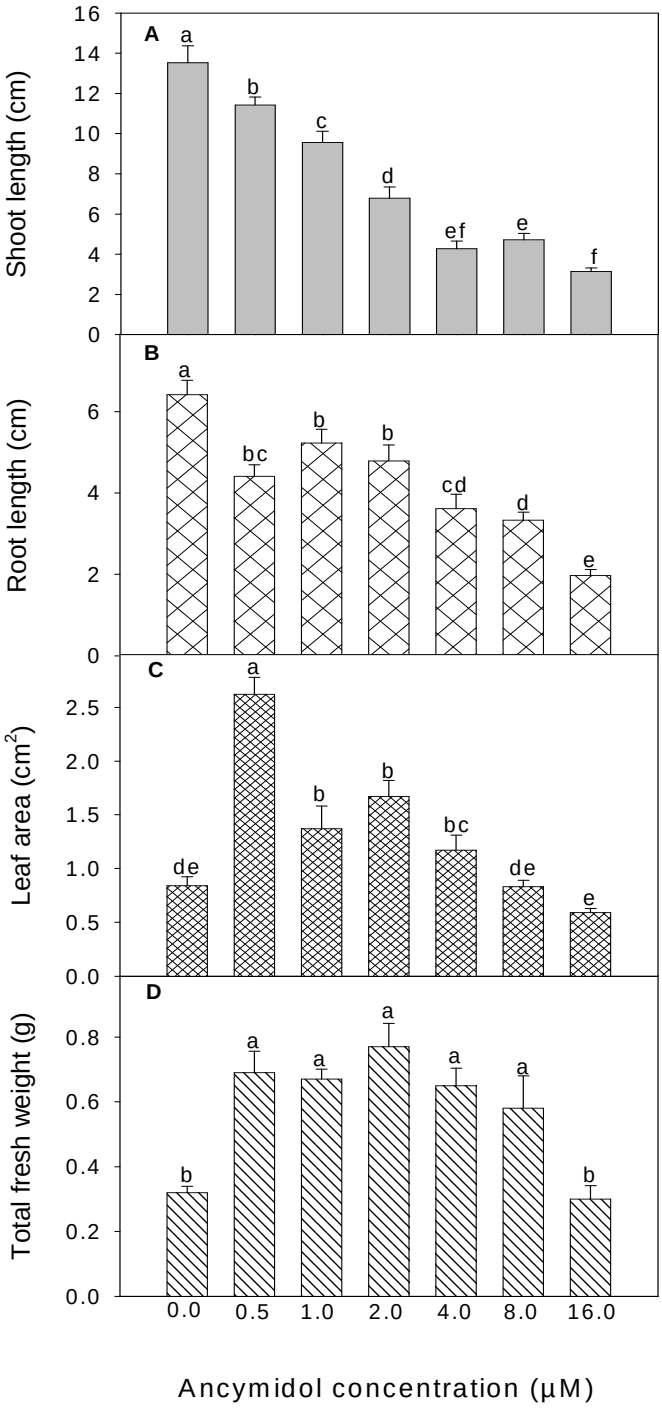


Figure 4.2: Effect of ancymidol concentrations on shoot length (**A**), root length (**B**), leaf area (**C**) and total fresh weight (**D**) in *D. erectum*. Bars with different letters indicate significant differences between treatments (P = 0.05, n = 15).

4.4.1.2 Effects of activated charcoal and sucrose

All AC and sucrose treatments failed to promote corm development *in vitro* even when the cultures were left in the same media for six months, cultures only continued forming shoot clusters (**Figure 4.3**). Sucrose is required for corm induction in *Gladiolus* **(DANTU and BHOJWANI, 1995)** and *Dierama luteoalbidum* **(MADUBANYA et al., 2006)**, bulb formation in *Brodiaea* species **(ILAN et al., 1995)** and *Nerine sarniensis* **(VISHNEVETSKY et al., 1997)**. However, in this study sucrose did not promote corm induction. Similar results were obtained in micropropagation of four *Watsonia* species, where sucrose promoted corm formation only in *W*. *vanderspuyiae* but not in *W*. *gladioloides*, *W*. *lepida*, and *W*. *laccata* **(ASCOUGH et al., 2007)**. Carbohydrate depletion in a plant results in down regulation of mRNAs for storage and utilization **(KOH, 1996)**. It is therefore reasonable to assume that abundant sugar levels could exert an opposite effect resulting in storage organ formation. However in micropropagation of *D*. *erectum*, elevated sucrose levels (up to 5% w/v) did not promote corm formation. According to **LI and ZHANG (2003)**, the expression and abundance of sucrose metabolism genes present, among other factors, are responsible for storage organ formation in the plant.

AC has also been reported to be involved in storage organ induction in *Lilium* species **(BACCHETTA et al., 2003)**, *Allium sativum* **(MOHAMED-YASSEEN et al., 1993)** and *Narcissus tazetta* **(STEINITZ and YAHEL, 1982)**. However, AC did not influence corm induction of *D*. *erectum*. Similar results were obtained by **MADUBANYA et al. (2006)** where AC inhibited corm formation in

D. luteoalbidum at a high (8%) sucrose concentration. There are many factors responsible for success or failure of *in vitro* storage organ formation: the genetic make-up of the plant, media additives, culture conditions or the concentrations of endogenous auxins and cytokinins present in the plant **(ASCOUGH et al., 2008b)**. Since shoots used for this experiment were obtained from the medium supplemented with 1.0 µM BA, this may have had a carry-over effect in this experiment, allowing plants to produce shoot clusters on hormone-free medium (as seen in **Figure 4.3**) instead of corms. Negative effects of BA in micropropagation were also reported by **WERBROUCK et al. (1996)** and **BAIRU et al. (2008)**.

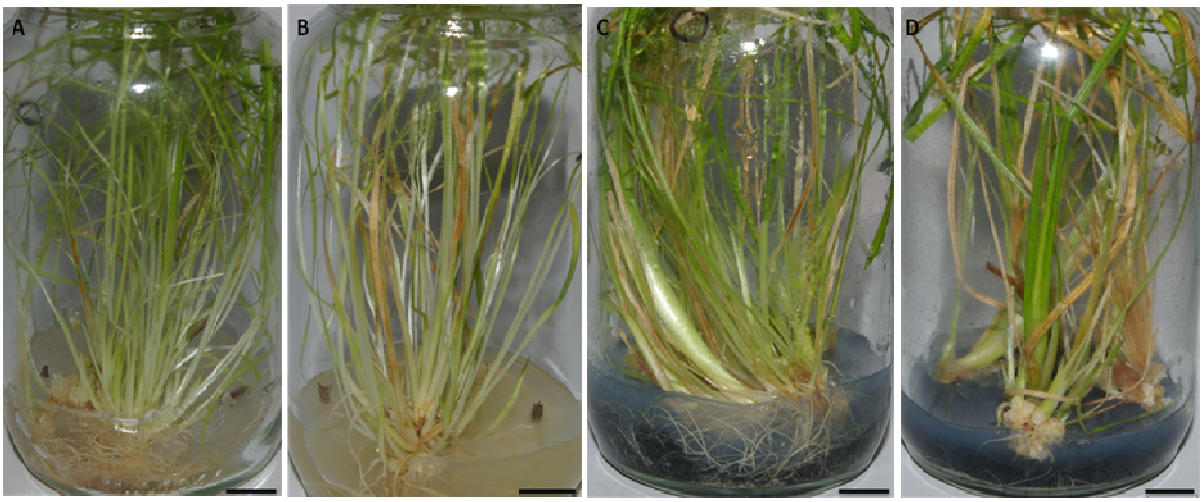


Figure 4.3: *D. erectum* cultured on various concentrations and combinations of AC and sucrose. **A** = control, **B** = 5% sucrose, **C** = 0.1% AC + 4% sucrose, **D** = 0.6% AC.

4.4.2 Effects of auxins on rooting

Due to unsuccessful corm induction *in vitro*, an efficient protocol for rooting of *D. erectum* had to be developed to facilitate *ex vitro* acclimatization. **Figure 4.4** shows the effects of IAA, IBA and NAA on rooting of *D. erectum*. The highest number of roots (2.10 ± 0.38 roots per explant) was obtained with the medium containing 0.5 µM IBA after 4 weeks (**Figure 4.4A**). When cultures were left under similar conditions for 8 weeks, the number of roots in all media types increased and the highest number of roots (2.93 ± 0.50 roots per explant) was found in the medium containing 0.5 µM NAA (**Figure 4.4B**), however this was not significantly different from other treatments except the control.

Different concentrations of auxins had various effects on root length. The longest roots (2.63 ± 0.39 cm) developed in the medium supplemented with 1.0 µM IAA after 4 weeks in culture (**Figure 4.4C**). When cultures were left under the same conditions for 8 weeks, there was an increase in root length. The longest roots (4.86 ± 0.38 cm) were obtained in the PGR-free medium and in the medium supplemented with 1.0 µM IBA. The observation that *D. erectum* was able to do well in the PGR-free medium in terms of root length suggests that this plant does not require an auxin for better root length. At all NAA concentrations, stunted roots were produced (**Figures 4.4D** and **4.5 E-F**).

IBA is used for plant propagation because of its efficacy in stimulation of adventitious roots. The observation that IBA is more efficient than IAA at inducing roots (as observed in **Figure 4.4A**) may be explained by its stability

against catabolism and inactivation by conjugation **(NISSEN and SUTTER, 1990)**. Moreover, IBA is conjugated and metabolised into IAA **(SMULDERS et al., 1990)**, meaning that it can be stored and slowly released when needed by the plant, whereas IAA is unstable and more vulnerable to auxin-oxidase **(DE KLERK et al., 1999)**.

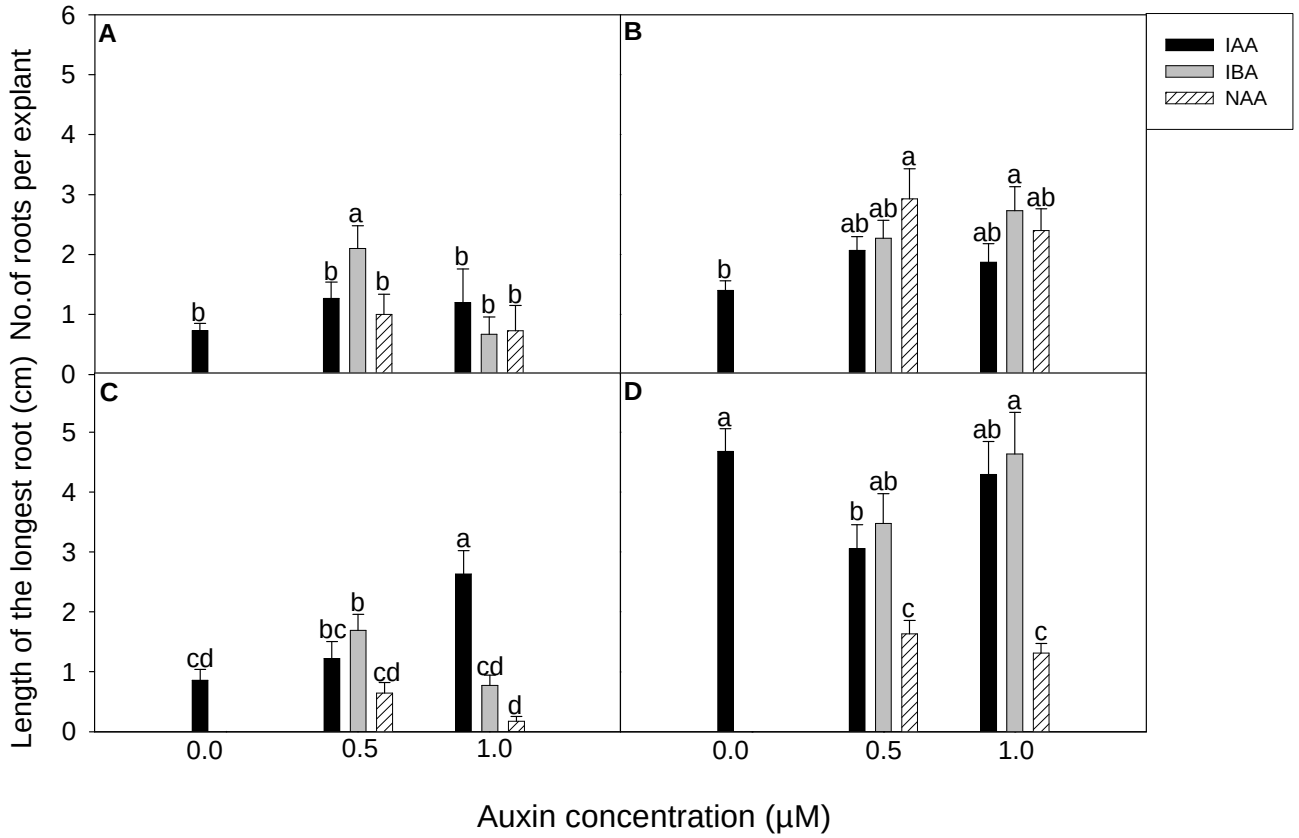


Figure 4.4: Effects of auxin types and concentrations on root production after 4 (**A**) and 8 weeks (**B**); root length after 4 (**C**) and 8 weeks (**D**). Bars with different letters indicate significant differences between treatments (*P* = 0.05, n = 21).

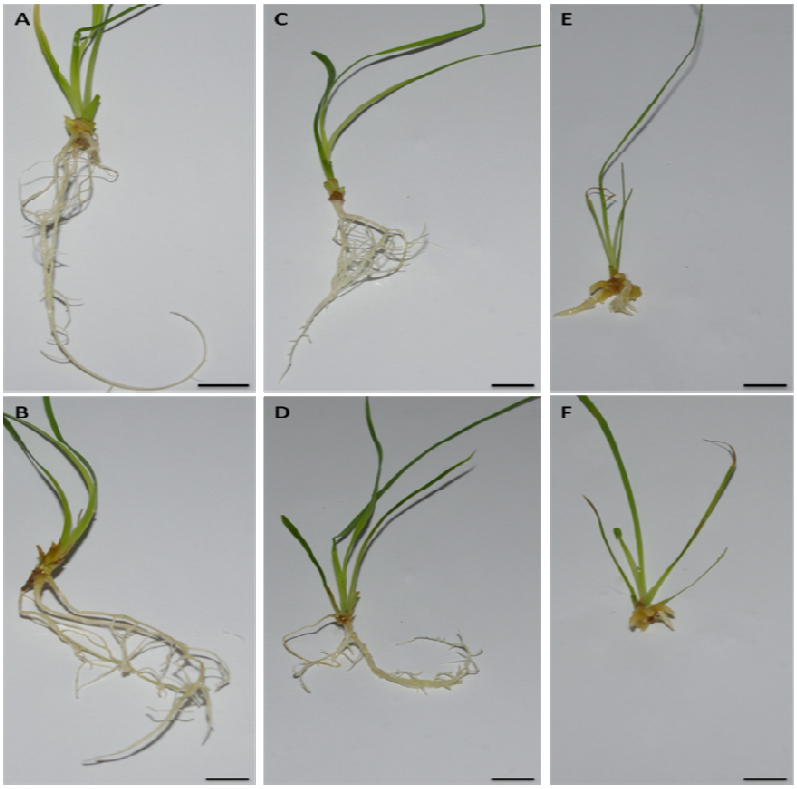


Figure 4.5: Effects of auxin types and concentrations on root length. **A-B** = IAA, **C-D** = IBA, **E-F** = NAA, **A-E** = 0.5 µM, **B-F** = 1.0 µM. Bar = 1.0 cm

NAA has inhibitory side effects and induces fewer roots *in vitro* than other auxins such as IAA and IBA **(DE KLERK et al., 1997)**. This seems to be also true for *D. erectum* (**Figure 4.4A**, **C** and **D**) where few roots were formed and root elongation was inhibited. This may be attributed to the stability and persistence of NAA in the tissue in its free form, resulting in stunted root growth **(DE KLERK et al., 1997)**.

The efficacy of different auxins depends on the affinity for the auxin receptor proteins involved in rooting, concentration of the auxin reaching the competent cells, the amount of endogenous auxin or the stability of the applied auxins

(FOGAÇA and FETT-NETO, 2005). Moreover, NAA is only conjugated, IAA is both conjugated and oxidized and IBA is conjugated and metabolized into IAA **(SMULDERS et al., 1990)**. This could explain the observed differences between auxin responses in the present study.

4.4.3 Acclimatization of plantlets in the *ex vitro* environment

Data presented on **Table 4.1** shows that the largest corms were obtained from plants previously cultured on MS medium supplemented with 0.5 µM IBA. The highest plant survival percentage was also obtained in this treatment. However, the corm diameter value obtained in this treatment was not significantly different from other treatments attempted in this experiment (including the control) except for the 1.0 µM NAA treatment. The use of auxins in micropropagation may to some extent, promote storage organ formation. For instance, NAA was necessary in bulb induction of *Lilium rubellum* **(NIIMI and ONOZAWA, 1979)** and IAA, IBA and NAA were found to be effective in bulb induction of *Hyacinthus orentalis* **(PIERIK and STEEGMANS, 1975)**. However, corms were formed in all the treatments with *D*. *erectum* (including the control) during acclimatization (**Figure 4.6**), suggesting that corm formation in this plant was not entirely dependent on the type and concentrations of applied auxins, but on various environmental factors that the plants were exposed to *ex vitro.* Low survival percentage in the 1.0 µM NAA treatment may be due to the poor root development (stunted roots) associated with it. Similar results were obtained by **ASCOUGH et al. (2007)** where NAA negatively affected *ex vitro* survival of *Watsonia* species. Plants which root well *in vitro* generally acclimatize faster

and are more resistant to pathogens **(WERBROUCK et al., 1996)** thus the high survival percentage observed with plants pre-treated with 0.5 µM IBA.

Table 4.1: Effects of auxin types and concentrations on acclimatization of *D. erectum*

Treatments (µM)	Corm diameter (cm)	% Survival
0.0	0.39 ± 0.033^{ab}	60
0.5 IAA	0.43 ± 0.035^{ab}	64
1.0 IAA	0.39 ± 0.026^{ab}	51
0.5 IBA	0.45 ± 0.026^{a}	73
1.0 IBA	0.44 ± 0.031^{ab}	60
0.5 NAA	0.37 ± 0.030^{ab}	47
1.0 NAA	0.35 ± 0.023^{b}	40

Values with different letters indicate significant differences between treatments ($P = 0.05$) based on DMRT (n = 15).

4.5 SUMMARY

- *In vitro* corm induction was unsuccessful; however, plants formed corms during a two-month acclimatization period.
- Ancymidol application decreased shoot and root length significantly but resulted in maximum leaf area (2.62 ± 0.66 cm^2) at 0.5 µM and increased the plant's total fresh weight at 0.5 to 8.0 µM.
- All sucrose and AC treatments were not successful in corm induction.

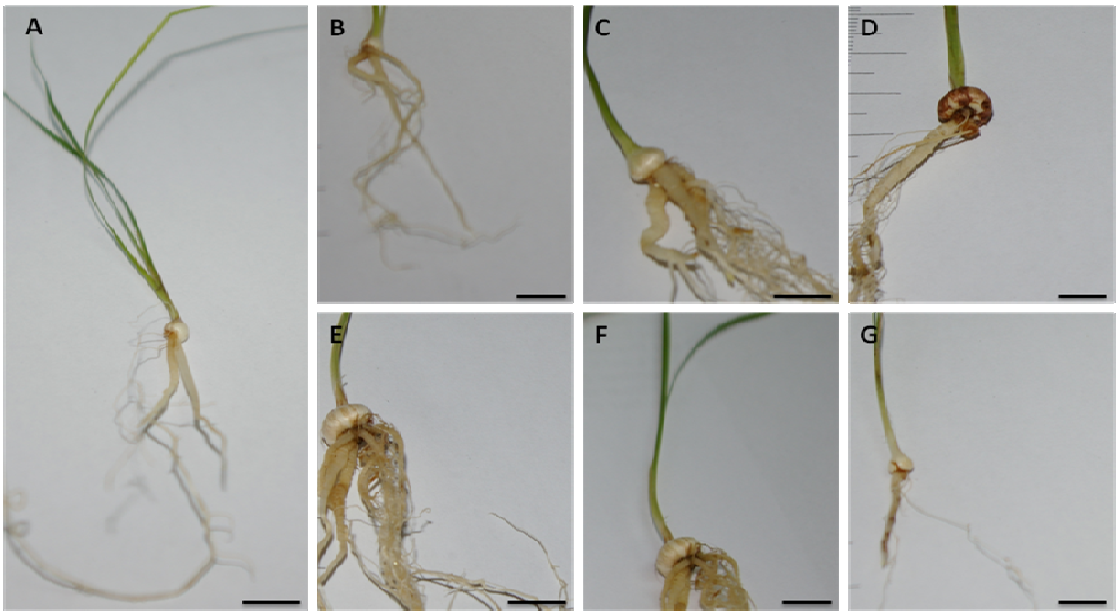


Figure 4.6: Effects of type and concentration (µM) of auxins on corm formation in *D. erectum*. **A** = Control, **B** = 0.5 IAA, **C** = 1.0 IAA, **D** = 0.5 IBA, **E** = 1.0 IBA, **F** = 0.5 NAA, **G** = 1.0 NAA. Bar = 1.0 cm.

Figure 4.7: Two-month-old acclimatized plants in the greenhouse.

- The maximum number of roots (2.93 ± 0.50 roots per explant) was obtained in the medium supplemented with 0.5 µM NAA after 8 weeks.
- The maximum root length (4.68 ± 0.38) was found in the hormone-free medium after 8 weeks.
- The largest corms (0.45 ± 0.026 cm in diameter) and highest plant survival percentage (73%) were obtained in plants pre-treated with 0.5 µM IBA.
- Plants were successfully acclimatized in a 1:1 ratio of soil:vermiculite (**Figure 4.7**).

CHAPTER FIVE:
GENERAL CONCLUSIONS

An efficient protocol for *in vitro* propagation of *Dierama erectum* was successfully developed. Seed decontamination was achieved with the use of either 0.2% $HgCl_2$ or a combination of 2.5% NaOCl and 1% Benlate®. However, since heavy metals such as mercury are toxic, the recommended decontaminating agents for *D. erectum* seeds are 2.5% NaOCl plus 1% Benlate®. Following an experiment investigating the regenerative capacity of different explants, it was found that hypocotyl sections had more regeneration potential than the root and leaf explants when exposed to various BA concentrations. The use of other PGRs besides BA could bring about regeneration in both leaf and root explants if further investigated.

In an experiment evaluating the effects of different types and concentrations of cytokinins (BA, *m*T, KIN and Z) with or without NAA on shoot multiplication, hypocotyl explants produced shoots with all the treatments. Shoot multiplication with BA application was generally high from 1.0 µM to 5.0 µM especially when combined with 1.0 µM NAA. Maximum shoot production (3.20 ± 0.22 shoots per explant) was obtained with the addition of 2.0 µM BA and 1.0 µM NAA in the growth medium. These results indicated that with the use of BA in the micropropagation of *D. erectum*, NAA is necessary for maximum shoot production.

An increase in *m*T concentrations in the presence of 2.0 µM NAA generally resulted in increased shoot multiplication. The maximum average shoot number

(3.09 ± 0.99) was obtained when 2.0 µM *m*T and 2.0 µM NAA were present in the medium. It was evident in this experiment that maximum shoot production of *D. erectum* with *m*T requires higher NAA concentrations (in this case 2.0 µM). The use of topolins in micropropagation of Iridaceae species has not previously been reported. These results revealed that topolins (especially *m*T) have the potential of inducing shoots in species of this family.

Addition of KIN without NAA in the medium generally increased shoot multiplication but this was only up to 5.0 µM. A combination of 2.0 µM KIN and 0.5 µM NAA resulted in maximum shoot production (3.67 ± 0.81 shoots per explant). Although KIN is considered a relatively weak cytokinin, the results obtained in this experiment revealed that KIN was an effective cytokinin especially when combined with low auxin concentrations such as 0.5 µM NAA. KIN was the second best cytokinin in the multiplication of *D. erectum* shoots.

Increasing the concentration of Z alone in the growth medium generally resulted in increased shoot multiplication. The optimum concentration was 1.0 µM Z giving an average shoot number of 4.20 ± 0.51 per explant. The high cytokinin activity of Z at concentrations as low as 1.0 µM was attributed to the *trans*-hydroxylation of the isoprenoid side chain of Z as well as the presence of a double bond at the 2,3-position of the side chain.

The micropropagation protocol developed in this study was further optimized by the manipulation of light regimes. Shoots grown in 16 h light at a 100 µmol m^{-2} s^{-1} light intensity produced an average of 12.73 ± 1.03 shoots per explant

after 8 weeks. This was about 3 times the multiplication rate obtained in the experiments investigating the effects of different combinations of cytokinins and NAA. It is believed that dark periods (8 h darkness) may favor synthesis and accumulation of substances which in turn are beneficial for growth during 16 h light periods.

The use of ancymidol, sucrose and activated charcoal did not yield any corms *in vitro*. This failure was possibly due to the carry-over effect of BA (since the plants were previously multiplied in 1.0 µM BA) allowing plants to continue producing shoot clusters instead of corms. The best auxin for rooting was IBA with 1.0 µM giving a higher number of roots (2.73 ± 0.40 roots per explant) after 8 weeks. This treatment was also associated with long roots (4.63 ± 0.70 cm). Shoots pre-treated with 0.5 µM IBA produced larger corms (0.45 ± 0.026 cm in diameter). It also showed the highest plant survival percentage (73%) after a 2 months acclimatization period. The efficiency of IBA in root induction is attributed to its stability against catabolism and inactivation by conjugation.

In conclusion, the recommended protocol for the micropropagation of this species is seed decontamination in 2.5% (v/v) NaOCl then in 1.0% w/v Benlate, seed germination on $1/10^{th}$ strength MS medium for 21 days, inoculation of hypocotyl explants into culture jars containing 1.0 µM BA in a 16 h photoperiod and PPFD of 100 µmol m^{-2} s^{-1}, rooting plants in MS medium supplemented with 1.0 µM IBA followed by acclimatisation of plants in the mist house for 2 weeks and transfer to the greenhouse. The first cycle which involves seed decontamination, germination and culture of explants on MS medium takes 3

months. If this protocol is employed the final yield of plants acclimatised from one explant will be about 15137 plants per year.

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