ORGANOGENESIS AND GENETIC TRANSFORMATION OF *DIERAMA ERECTUM* HILLIARD

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DOCTOR OF PHILOSOPHY

Research Centre for Plant Growth and Development

School of Life Sciences

University of KwaZulu-Natal, Pietermaritzburg

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STUDENT DECLARATION

Thesis title: Organogenesis and genetic transformation of *Dierama erectum* Hilliard

I, MOTSELISI JANE KOETLE, student number – 208523555, declare that:

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DECLARATION BY SUPERVISORS

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

Student's Full Name: Motselisi Jane Koetle

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

SUPERVISOR: PROFESSOR J VAN STADEN

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DETAILS OF CONTRIBUTION TO PUBLICATIONS that form part and/or include research presented in this thesis (including publications in preparation, submitted, *in press*, and published and details of the contributions of each author to the experimental work and writing of each publication).

PUBLICATION 1

KOETLE MJ, FINNIE JF, BALÁZS E, VAN STADEN J (2015) A review on factors affecting *Agrobacterium*-mediated transformation in ornamental monocotyledonous geophytes. South African Journal of Botany 98: 37-44.

Contributions: The article was written by the first author, reviewed by Prof Ervin Balázs. This work was supervised by Prof Jeffery F Finnie and Prof Johannes Van Staden.

PUBLICATION 2

KOETLE MJ, BASKARAN P, SOOS V, BALÁZS E, FINNIE JF, VAN STADEN J Optimizing factors affecting *Agrobacterium*-mediated transformation in *Dierama erectum.* Journal of Plant Biochemistry and Biotechnology (In preparation)

Contributions: Laboratory experiments were performed by the first author, Dr Baskaran and Dr Soos assisted with the experiments and analysis. The work was

supervised by Prof Ervin Balázs, Prof Jeffery F Finnie and Prof Johannes Van Staden.

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CONFERENCE PUBLICATIONS

DETAILS OF CONTRIBUTION TO CONFERENCES that form part and/or include research presented in this thesis:

1. 39th Annual Conference of the South African Association of Botanists (SAAB): 20-24 January 2013; Drakensberg, KwaZulu-Natal, SOUTH AFRICA.

   Title of Paper: **MJ KOETLE, P BASKARAN, JF FINNIE, J VAN STADEN (2013)** The role of different explants on the transformation efficiency in *Dierama erectum* Hilliard.

Signed:_____

ABSTRACT

Genetic improvement of ornamental geophytes especially the monocotyledonous plants; is often restricted by the failure of *Agrobacterium* to reach competent cells as well as a lack of efficient regeneration systems. Despite all these limitations, it has recently been shown that the use of efficient promoters, super-virulent strains, and the utilization of systems such as an agrobacterial monolayer, *Agrobacterium*-mediated pollen and seed transformation, floral dip method and SAAT will ensure success in the genetic transformation of ornamental monocotyledonous geophytes in the near future. In this thesis, an outline of factors affecting transformation of monocotyledonous geophytes is given. Special emphasis is laid on measures that have been employed to alleviate various difficulties. The need to develop somatic embryogenesis protocols for the ease of transformation is highlighted. In addition, perspectives in view of future research are also given. This information is crucial for biotechnological improvement of ornamental geophytes that are proving difficult to transform.

Experiments were designed to induce callus that would assist genetic transformation of *Dierama erectum* and to further understand the behaviour of this plant *in vitro*. With the aim of obtaining somatic embryogenesis, different concentrations of auxins (NAA and 2,4-D) at concentrations of 0, 0.5, 1.0, 2.0 and 2.5 mg L^{-1} were added to MS medium to induce callus. It was found that *D. erectum* could generate organogenic callus with NAA concentrations between 0.5 and 2.0 mg L^{-1}. The treatment that resulted in highest callus formation (2.50 ± 0.34 explants forming callus) was MS fortified with 1.0 mg L^{-1} NAA. Application of 2,4-D alone at all given concentrations did not induce callus, instead detrimental effects such as explant browning were evident.

Further investigations to obtain the best culture medium combination for callus induction was conducted by including combinations of PGRs (1.0 mg L^{-1} NAA + 0.1 mg L^{-1} BA, 1.0 mg L^{-1} NAA + 0.1 mg L^{-1} *m*T, 1.0 mg L^{-1} 2,4-D + 0.1 mg L^{-1} *m*T, and 1.0 mg L^{-1} Picloram + 0.1 mg L^{-1} TDZ) together with varied sucrose concentrations (30, 35 and 40 g L^{-1}). Generally, the mean number of explants producing callus was very low when compared to NAA treatments alone. Instead of callus formation, a combination of 1.0 mg L^{-1} 2,4-D + 0.1 mg L^{-1} *m*T encouraged shoot regeneration. Callus induction was significantly deterred when NAA was combined with either BA or *m*T. It was therefore concluded that a clear explanation of the concepts behind somatic embryogenic callus induction could be better obtained by adopting molecular based studies. The rooting and acclimatization of regenerants obtained from organogenic callus was very successful since both root and shoot formation increased in the presence of 0.2 and 0.4 mg L^{-1} IBA.

The second major objective of this study was to optimize factors influencing the efficiency of *Agrobacterium*-mediated *GUS* expression, with the intention of forming a basic genetic transformation system for *D. erectum*. Experiments were conducted to investigate the effects of explant types, co-cultivation time, acetosyringone concentration, *Agrobacterium* concentration and different gene delivery systems. This study showed that better transformation efficiency (over 60%) could be achieved when ESAMs are used as explants rather than callus clusters. Use of hypocotyl explants did not result in any transformation event. Genetic transformation via SAAT proved to be the most efficient gene delivery system with about 40% transformation efficiency. The utilisation of this system together with ESAMs was also associated with multiple shoot formation during regeneration. It was hence concluded that the use of low frequency

ultra sound does enhance the efficiency of transformation in *D. erectum*. On the other hand, the use of organogenic callus from 1.0 mg L^{-1} NAA proved to be inefficient for transformation. However, the establishment of this organogenic callus does have a potential as means for rapid multiplication of *D. erectum* transformants. Another important factor is density of *Agrobacterium* inoculum. An OD_{600} of 1.6 was optimal for transformation resulting in about 60% transformation efficiency. An acetosyringone concentration of 50 mg L^{-1} improved transformation efficiency by 80%, although this was not significantly different from the control. Another crucial factor; co-cultivation duration was investigated. The study showed that transformation was high between 1 and 3 days, with the optimal co-cultivation time being 3 days giving rise to 75% transformation efficiency.

From the fore-mentioned experiments, plants were rooted and acclimatised. The genomic DNA was isolated and the PCR amplification results indicated that out of 17 plants which histochemically expressed *GUS*, six (6) T_0 transformants were *GUS* positive. These results indicated that *D. erectum* is amenable to *Agrobacterium*-mediated transformation. However after Southern blotting, it was revealed that the *GUS* gene was transiently expressed in all the transformed plants.

The last part of this study investigated the possibility of integrating the early flowering gene of interest; *BpMADS4* into the *D. erectum* genome. The conditions optimized for *GUS* gene integration were used in this section. After acclimatisation of putative transformants, plants were grown in alternating photoperiod regimes (LD or SD) to trigger flowering. However, no flower competent stage was observed over 6 months.

PCR amplification results showed that *BpMADS4* was integrated in one of the T_0 transformants yet again no early flowering was observed from this plant. It was speculated that the gene could not be expressed at functional level. An attempt to introduce *BpMADS4* through particle bombardment was a failure since the explants did not survive on the kanamycin-rich medium for selection. Despite disappointing results of failure to achieve early flowering, the molecular analysis of transformants showed that there is a possibility of introducing other genes of interest in *D. erectum*.

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LIST OF ABBREVIATIONS

2,4-D - 2,4-Dichlorophenoxy acetic acid

2-ME - 2-Mercaptoethanol

ANOVA - Analysis of variance

ARFs - Auxin response factors

BA - Benzyladenine

BAP - Benzylaminopurine

CaMV - Cauliflower mosaic virus

CDKs - Cyclin-dependent kinases

CMV - Cucumber mosaic virus

crtB - Bacterial photoene synthase

CTAB - Cetyltrimethylammonium bromide

DNA - Deoxyribonucleic acid

ESAMs - Embryonic shoot apical meristems

GUS – β-Glucuronidase

IBA – Indole-3-butyric acid

LD - Long day

LIccs - Capsanthin-capsorubin synthase

*m*T - *meta*-Topolin

NAA - 1-Naphthalene acetic acid

NaCl - Sodium chloride

OD - Optical density

PGRs - Plant growth regulators

PSY - Phytoene synthase

PVPP - Polyvinylpoly-pyrolindone

SAAT - Sonication-assisted *Agrobacterium*-mediated transformation

SD - Short day

SEM - Scanning electron microscope

Sm/Sp - Spectinomycin

T-DNA - Transfer DNA

TDZ - Thidiazuron

Ti - Tumour-inducing plasmid

CHAPTER 1

INTRODUCTION

1.1 Ornamental perennials and the horticulture industry

The global trade for ornamental perennials and garden plants is in excess of eight billion dollars per year **(PRAKASH, 2009)**. Over one billion ornamental plants are produced through micropropagation **(PRAKASH, 2009)**. These figures indicate that ornamental horticulture is a very important aspect of horticulture. Floriculture in turn is a big sector of ornamental horticulture. One feature of floriculture is that it encompasses cut flowers, pot plants and bedding plants. Unlike with agriculture; where crops such as maize and rice are planted yearly, floriculture is dynamic and consumers require new varieties regularly. Since this is a global industry in which many countries such as Colombia, Ecuador, Kenya, Israel and South Africa have developed large industries, it is important to consider programs aimed towards the development of genetically modified ornamental products **(CHANDLER and LU, 2005)**. Bulbous plants are not only desirable for their ornamental value, but also for their benefits in traditional medicine; which may sometimes render them endangered in the wild. Thus the applications of micropropagation techniques and other biotechnological tools remain important.

1.2 The possibility of genetically transforming Iridaceae species

The Iridaceae is a family of perennial, herbaceous and bulbous plants. It is one of the most important families in horticulture and includes more than 2000 species

(GOLDBLATT and MANNING, 2008). *Dierama erectum* is included in the Iridaceae as one of the most valuable plants with a high potential to be developed into an ornamental plant **(SCOTT-SHAW, 1999)**. Some of its outstanding features are large-sized, magenta-pink flowers.

The challenges facing *Agrobacterium*-mediated transformation of monocotyledonous geophytes such as *Dierama* are well known (See **Chapter 2**). Despite this, some related species have reportedly been successfully transformed via *Agrobacterium* systems of gene delivery. For instance, cormels and shoot-tips of a close relative; *Gladiolus* were transformed **(BABU and CHAWLA, 2000; KAMO et al., 2010)**. Other examples include; *Agapanthus praecox* **(SUZUKI et al., 2001)**, *Narcissus tazzeta* **(LU et al., 2007)** and *Allium sativum* **(KONDO et al., 2010)**. In most of these reports, only basic genetic transformation procedures have been outlined without introduction of genes of interest. Some of the traits that can be modified in monocotyledonous geophytes include increased vase life, flower colour enhancement, fragrance and male and female sterility among others.

Dierama is a genus belonging to the Iridaceae. *D. erectum* is found mostly in wet grasslands **(HILLIARD and BURTT, 1991)**. Its corms are used as a remedy for stomach ailments. However, it is known more for its ornamental value than medicinal use. In fact, a successful introduction of *Dierama* species as garden plants has been recorded as early as 1825 in Britain and France **(HILLIARD and BURTT, 1991). SCOTT-SHAW (1999)** emphasised on the high economic and horticultural potential of *D. erectum* as it has a very attractive foliage and beautiful erect flowers. The present research was designed to investigate fundamental factors contributing to successful

basic *Agrobacterium*-mediated genetic transformation in *D. erectum*. This approach will allow for future modifications such as flower enhancement (colour and size) and high throughput production of medicinally important phytochemicals.

1.3 Background to the research problem

The establishment of different micropropagation techniques for various *Dierama* species **(PAGE and VAN STADEN, 1985; MADUBANYA et al., 2006 and KOETLE et al., 2010)** provided the basis for potential development of new cultivars. In as much as genetic modification of *D. erectum* and many other geophytes are important for improvement of their ornamental traits; more studies focusing on the mechanisms that favour mass propagation, somatic embryogenesis and particularly genetic transformation are required for a better understanding of these geophytes. For instance, in ornamental geophytes the timing of transition from vegetative to the flowering phase is critical as it determines the plant's growth cycle **(BERNIER et al., 1993; SCORTECCI et al., 2001)**. Most geophytes must pass through a long juvenile phase of vegetative development before flowering **(LIN et al., 2003)**. For example, after seed germination; *Dierama* seedlings remain in a juvenile, floral incompetent stage which can last 3 to 4 years. In view of this problem, the development of a basic genetic transformation protocol is recommended. This will lay a foundation for important features such as shortening of juvenility in *D. erectum,* manipulation of the corolla size and colour which will be of immense biotechnological interest and horticultural benefit. The study will not only be important for the development of ornamental traits in *Dierama*, but will also afford basic in-depth biological studies , as well as adding more details to the existing knowledge on the genus *Dierama*.

Controlling the environment in which plants are grown enables manipulation of different variables that affect important processes such as genetic transformation. Understanding the process of callus induction and somatic embryogenesis in *D. erectum* is one of the first steps towards molecular based studies of gene integration in the genome. Therefore, somatic embryogenesis can be a vital tool needed to accelerate genetic transformation programs for commercial purposes.

1.4 Objectives of the study

The general aim of this study was to develop a basic protocol for callus induction so as to assist the subsequent genetic transformation process of *D. erectum in vitro*. Manipulation of plant growth regulators (PGRs); cytokinins and auxins, were done to obtain callus. Different systems of gene delivery and standardization of different factors involved in genetic transformation of *D. erectum* were employed.

1.4.1 Specific objectives

Callogenesis and regeneration experiments focusing on:

- Callus induction through the use of different combinations of auxins and cytokinins;
- Macroscopic evaluation of calli; and
- Rooting and acclimatization of regenerants

The genetic transformation section of this study was aimed at investigating:

- Sensitivity of hypocotyl explants and callus clusters to antibiotics;

- Evaluation of factors (explant type, co-cultivation time, acetosyringone concentration and the gene delivery systems) affecting the *GUS* gene delivery into the plant genome;
- Integration of an early flowering gene; *BpMADS4* into the *D. erectum* genome;
- Evaluating transgenic lines by histochemical *GUS* assay;
- Analysis of putative transformants from the selection medium;
- Confirming genetic transformation via PCR and Southern blotting; and
- Phenotypic analysis of acclimatized transgenic plants

1.5 General overview of this thesis

CHAPTER 1: General introduction

The thesis is arranged in six (6) Chapters, 3 of which are research Chapters. The current section gives the background information, rationale for the study and its objectives.

CHAPTER 2 Literature review

This Chapter outlines factors affecting callus induction (with emphasis to the Iridaceae) and transformation of monocotyledonous geophytes. Special emphasis is laid on measures that have been employed to alleviate various difficulties in genetic transformation of monocotyledonous geophytes. The need to develop somatic embryogenesis protocols for the ease of transformation is highlighted. In addition, perspectives in view of future research are also given. This information is crucial for

biotechnological improvement of ornamental geophytes that are proving difficult to transform.

CHAPTER 3 Callus induction and organogenesis

This is an investigation on callus induction, shoot and root formation from callus in *D. erectum*, under the influence of different cytokinins and auxins.

CHAPTER 4 *Agrobacterium*-mediated transformation: investigating factors affecting *GUS* gene expression

This entails a basic *Agrobacterium*-mediated genetic transformation of *D. erectum*. Various factors underlying this method of transformation are investigated for optimization of an efficient genetic modification system.

CHAPTER 5 Transformation of *Dierama erectum* with the early flowering *BpMADS4* gene

Various attempts to introduce an early flowering gene into *D. erectum* were investigated via sonication-assisted *Agrobacterium*-mediated transformation and biolistic approaches.

CHAPTER 6 General conclusions

This provides an analysis of the implications of the findings in this research study.

CHAPTER 2
LITERATURE REVIEW

2.1 Callogenesis and Regeneration in Geophytes

2.1.1 Introduction

Micropropagation applications for ornamental geophytes, are mostly aimed at mass propagation, germplasm conservation and forming a solid foundation for developing new cultivars through recombinant DNA techniques. These among others include developing cultivars for disease and viral resistance **(VAN EMMENES et al., 2008; KAMO et al., 2010)**, colour and scent enhancement or high throughput production of medicinally recognized phytochemicals **(COLLING et al., 2010)**.

While the above facts remain pertinent, micropropagation protocols of many other geophytes have been brought forward for the ease of genetic transformation on species of interest **(ASCOUGH et al., 2009; REINTEN et al., 2011)**. Although excellent protocols on micropropagation of ornamental geophytes have been achieved and published (especially of monocotyledonous species), there are only a few reports on their genetic modification **(Table 2.1)**. Therefore, as the interest in developing new cultivars increases, a review on the requirements to achieve successful genetic transformation of these geophytes will be of considerable value.

In some plants, regeneration via callus is generally considered advantageous over direct regeneration especially in plant genetic transformation procedures since selection of homogenous transgenic plants is easily attainable **(HONG et al., 2007)**.

One of the major pathways of *in vitro* cell differentiation is organogenesis. This encompasses the formation of vegetative shoots, roots and floral structures **(BOLTENKOV and ZAREMBO, 2005)**. Therefore, a series of events leading to shoot formation is known as shoot organogenesis. The most common way of inducing callus and subsequently organogenesis is the adjustment of composition and concentration of plant growth regulators in the growth medium **(CHEN et al., 2003).**

2.1.2 Callus as a target explant for *Agrobacterium*-mediated genetic transformation

Callus induction in geophytes like in many other plants, is aimed towards mass propagation **(ASCOUGH et al., 2009)** and high throughput secondary metabolites production. For instance in *Iris ensata*, it was through callus production that flavones uncharacteristic of intact plants were identified **(BOLTENKOV et al., 2005)**. In the case of *Crocus sativus*, callus induction was done for mass production of the commercially important crocin, crocetin, picrocrocin and safranal **(CHEN et al., 2003; AHAMAD et al., 2014)**. This is also true for genetic transformation. The use of callus in transformation follows a pattern as illustrated in **Fig. 2.1** by **SHRAWAT and LÖRZ (2006).**

Embryogenic callus derived from japonica rice (*Oryza sativa* L.) was reported to be the best target explant for *Agrobacterium*-mediated transformation due to its active cell division **(HIEI et al., 1997)**. From then, the use of callus in *Agrobacterium*-mediated transformation systems was extended to ornamental geophytes such as *Agapanthus praecox* **(SUZUKI et al., 2001; SUZUKI and NAKANO, 2002)** and grasses like *Zoysia sinica* **(LI et al., 2006)**. Most recently, transformation of *Gladiolus*

cv. ‘Advanced Red’ was established through the embryogenic callus system for regeneration **(ZIYOU et al., 2011)**. Callus was also used in experiments investigating the effectiveness of GUBQ2 and GUBQ4 ubiquitin promoters from *Gladiolus* **(KAMO et al., 2011)**. Stable overexpression of the *LIccs* (capsanthin-capsorubin synthase) gene was also reported using callus tissue of *Iris germanica* **(JEKNIC et al., 2012)**. Flower color alteration in *Iris germanica* was also attained by using callus and the resulting plantlets showed prominent ectopic expression of the *crtB* (bacterial phytoene synthase) gene isolated form *Pantoea agglomerans* **(JEKNIC et al., 2014)**. These reports and many others, demonstrate the importance of callus as a useful target for genetic transformation in geophytes.

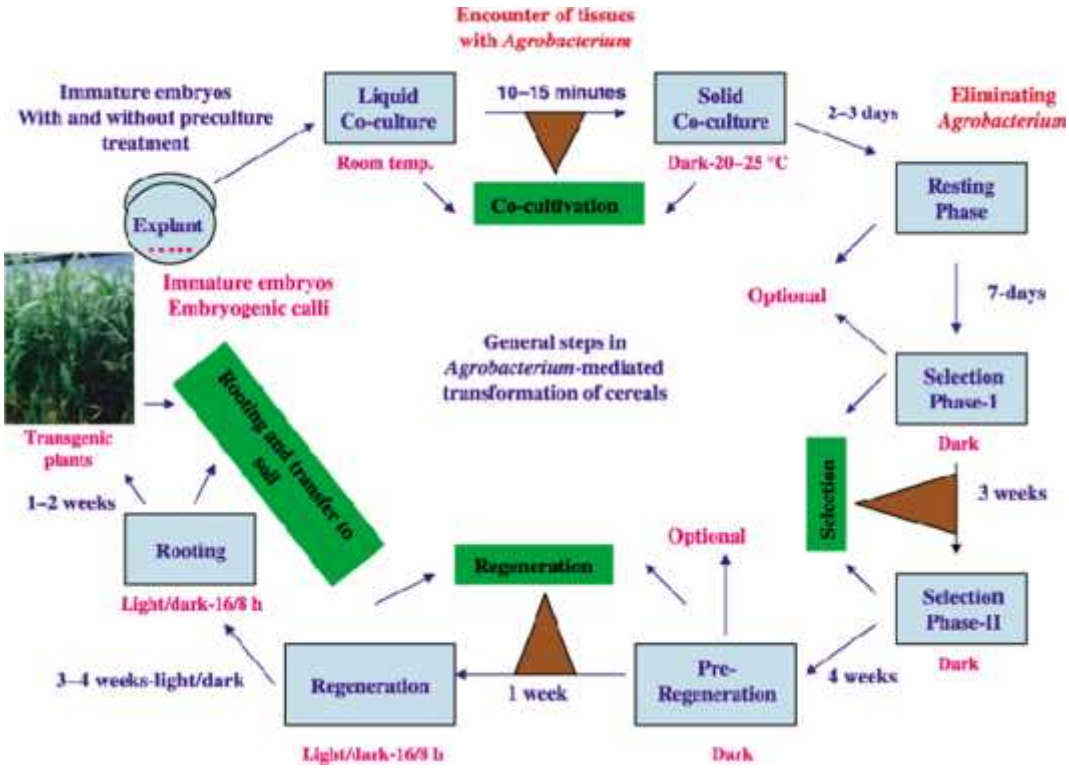

Figure 2.1 General scheme for *Agrobacterium*-mediated transformation using callus **(SHRAWAT and LÖRZ, 2006)**

2.1.3 Requirements for callus initiation in the Iridaceae

2.1.3.1 Plant growth regulators

The requirements for callus initiation and hence subsequent plant regeneration from monocotyledonous bulbous plants are less understood **(MEMON et al., 2012)**. Generally, an intermediate ratio of auxin and cytokinin promotes callus induction, a high auxin-to-cytokinin induces root regeneration and a high cytokinin-to-auxin ratio promotes shoot production. In species like *Gladiolus*; various reports have revealed that the best callusing occurs in the presence of BAP, NAA or 2,4-D **(AFTAB et al., 2008)**. A combination of 5.3 µM NAA and 4.4 µM BA promoted callus formation from hypocotyl explants in six species of *Babiana* **(MCALISTER et al., 1998)**. Leaf explants of *Crocus sativus* produced callus that generated somatic embryos when inoculated in medium containing 10 µM BA and 0.5 µM 2,4-D **(RAJA et al., 2007)**. Callus production and somatic embryogenesis was observed when 4.5 µM 2,4-D and 19 µM kinetin were used in three *Crocus* species namely *C. cancellatus*, *C. caspius*, and *C. michelsonii* **(KARAMIAN, 2004)**. In *Dierama latifolium*, callus was formed from corm explants on the MS medium fortified with 2.7 µM NAA **(PAGE and VAN STADEN, 1985)**. It is clear that most somatic embryogenesis protocols employ 2,4-D. This is because of its high efficiency for induction of embryogenic response. This response is indicative of its action as an effective stressor, triggering embryogenic patterns of development in plant cells **(GAJ, 2004)**. In some cases, media supplemented with concentrations varying from 1 to 10 mg L^{-1} NAA, 0.5 to 2.0 mg L^{-1} 2,4-D or 1.0 mg L^{-1} Picloram have also been reported to be necessary for callus initiation in *Gladiolus* **(KAMO et al., 2010)**.

It is evident from these reports that the requirements for callusing in the Iridaceae are obscure and vary from species to species and from one plant growth regulator to another. Recent studies show that at the molecular level, callus induction is dependent on modulation of plant growth regulator signaling; especially of auxins and cytokinins **(IKEUCHI et al., 2013)**. Several regulators such as auxin response factors (ARFs) and cyclin-dependent-kinases (CDKs), play a major role in callus induction **(IKEUCHI et al., 2013)**. It is therefore evident that the hormonal and developmental pathways in plants are inter-connected at multiple levels and molecular-based studies can bring about better understanding of the requirements for callus induction.

2.1.3.2 Carbon sources

Different sugars are included in the plant tissue culture media to serve as a source of energy and for creating the appropriate osmotic conditions for cell growth **(GAJ, 2004)**. Sucrose is the most widely used sugar in tissue culture and some studies have indicated that it can be a key factor in determining the morphogenic pathway of a given plant. A relatively high sucrose concentration (9% w/v) favoured callus induction in *Lilium longiflorum* **(ARZATE-FERNÁNDEZ et al., 1997)**. Substituting sucrose with maltose enhanced callus formation and its regeneration in *Triticum aestivum* **(MENDOZA and KAEPPLER, 2002)**. The highest frequency of callus induction was observed when 3% w/v sorbitol was used instead of sucrose in soybean cultures **(SANRAM et al., 2003)**. In some reports however, increasing the sucrose concentration proved to be detrimental to callus formation and regeneration especially in *Phalaenopsis* (orchid), where an increase in concentration from 1% to 2% w/v resulted in complete necrosis of the explant. This was attributed to high osmotic stress

inflicted by sucrose **(TOKUHARA and MII, 2001)**. These reports suggest that varying the concentration and type of carbon source can have an effect on callus induction and subsequent regeneration of plantlets.

Micropropagation protocols of many other plants have been brought forward for the ease of genetic transformation on species of interest. Although excellent protocols on micropropagation of ornamental geophytes **(Table 2.1)** have been and are still being published **(HUSSEY, 1977; ASCOUGH et al., 2009; MOYO et al., 2011)**, there are only a few reports on their genetic modification **(Table 2.2)**.

Table 2.1 Examples of ornamental geophytes in which micropropagation protocols were established without genetic transformation

Species name	Explant type	*PGR used	Growth response	References
Albuca bracteata, A. nelsonii	Bulb segments and peduncle sections	BA, *m*TR, NAA, IAA, GA_3 ABA, methyl jasmonate	Direct organogenesis, peduncle explants produced callus in media containing 0.5 mg L^{-1} *m*TR + 0.5 mg L^{-1} NAA	**ASCOUGH and VAN STADEN (2010)**
Brunsvigia undulata	Twin scales	BA, NAA	Direct organogenesis (bulb production)	**RICE et al. (2011)**
Crinum variabile	Twin scales	BA, NAA	Direct organogenesis (shoot and bulb production)	**VAN STADEN et al. (2001)**
Crocus cancellatus	Sprouted corm segments	BA, KIN, 2,4-D, IAA, NAA	Embryogenic calli production	**KARAMIN and EBRAHIMZADEH (2001)**

Cyrtanthus clavatus and *C. spiralis*	Twin scales	BA, NAA, 2,4-D	Direct organogenesis (shoot and bulb production)	**MORÁN et al. (2003)**
Dierama erectum	Hypocotyls	BA, KIN, *m*T, Z	Direct shoot production	**KOETLE et al. (2010)**
Drimia robusta	Leaves	BA, TDZ, IAA, IBA, *m*T, *m*TR	Direct organogenesis (multiple shoot and root production)	**BASKARAN et al. (2013)**
Eucomis zambesiaca	Leaves	NAA, IAA, IBA, 2,4-D, BA, *m*T, zeatin	Direct organogenesis (bulblet production)	**CHEESMAN et al. (2010)**
Merwilla plumbea	Leaves	Picloram, TDZ, 2,4-D	Somatic embryogenesis (production of friable embryogenic callus and somatic embryos)	**BASKARAN and VAN STADEN (2012)**
Romulea multiflora, R. sabulosa	Roots, leaves, hypocotyls	NAA, paclobutrazol, BA, NAA, IAA, IBA	Direct organogenesis (shoot induction)	**ASCOUGH et al. (2011)**
Sisyrinchium laxum	Roots, leaves, hypocotyls	NAA, paclobutrazol, BA, NAA, IAA, IBA	Direct organogenesis (shoot induction)	**ASCOUGH et al (2011)**

Tigridia pavonia	Twin scales	TDZ, Z, KIN, IAA, NAA, IBA	Direct organogenesis (multiple shoot production)	**KUMAR et al. (2012)**
Tritonia gladiolaris	Roots, leaves, hypocotyls	NAA, paclobutrazol, BA, NAA, IAA, IBA	Direct organogenesis (shoot induction)	**ASCOUGH et al(2011)**
Tulbaghia violacea	Flowers and shoots	BAP, NAA, kinetin	Direct organogenesis (multiple shoot production)	**PHELAN et al. (2007)**

*2,4-D = 2,4-Dichlorophenoxy acetic acid, ABA = Abscisic acid, BA = Benzyladenine, BAP = Benzylaminopurine, GA_3 = Gibberellic acid, IBA = Indole-3-butyric acid, KIN = Kinetin, *m*T = *meta*-Topolin, *m*TR = *meta*-Topolin Riboside, NAA = 1-Naphthaleneacetic acid, PGR = Plant growth regulator , TDZ = Thidiazuron, Z = Zeatin

Table 2.2 List of monocotyledonous geophytes successfully transformed genetically

Host plants	Explant types	*Gene delivery methods	Strains used	Objectives	Outcomes	References
Allium sativum L.	Callus	*Agr*	EHA101	To investigate factors affecting transformation	Temperature and co-cultivation time were important factors in transient expression of the *uidA* gene	**KONDO et al. (2010)**
Agapanthus praecox Willd.	Callus	*Agr*	EHA101, LBA4404	To compare effects of different *Agrobacterium* strains on transformation	Type of strain used affected transformation frequency. The most efficient strain was LBA4404	**SUZUKI et al. (2001)**
Gladiolus cvs American Beauty and Yellow Topaz	Shoot tips	*Agr*	LBA4404	To compare wounding effects on	The study highlighted the importance of pre-wounding explants before	**BABU and CHAWLA (2000)**

				explants before Agro-infection	commencement of transformation	
Gladiolus cvs. Jenny Lee and Peter Pears	Cormels	Particle bombard-ment		To develop cucumber mosaic virus resistant plants	Some transgenic plantlets were found to be resistant to CMV subgroup I and II	**KAMO et al (2010)**
Hyacinthus orientalis L.	Leaves	*Agr*	CBE21	To develop hyancinth plants resistant to *Fusarium culmorum* and *Botrytis cinerea*	Transgenic plants expressing thaumatin II gene were highly resistant to pathogenic fungus	**POPOWICH et al. (2007)**
Lilium cv. Acapulco	Callus	*Agr*	EHA101	The aim was to establish if *Agrobacterium*-mediated gene transfer is possible	Regenerated plantlets were verified to be transgenic by *GUS* histochemical assay and reverse PCR	**HOSHI et al. (2004)**

Lilium longiflorum Thunb	Callus	Particle bombard-ment		To develop plants resistant to cucumber mosaic virus	Transgenic plants obtained exhibited resistance against cucumber mosaic virus	**LIPSKY et al. (2002)**
Lilium longiflorum Thunb.	Bulb scales	*Agr*	EHA101, LBA4404	To develop a basic *Agrobacterium*-mediated gene transfer protocol	Results indicated that transformation in *Lilium* depends on the type of *Agrobacterium* strain used	**COHEN and MEREDITH (1992)**
Narcissus tazzeta L.	Leaves	*Agr*	LBA4404	To evaluate effects of various factors on transformation efficiency	Factors such as co-cultivation period of 3 days and acetosyringone concentration (100 µM) yielded efficient *GUS* expression	**LU et al. (2007)**

Ornithogalum thyrsoides Jacq. × *O. dubim* Houtt.	Leaves	*Agr*	AGL1	The study investigated resistance of transgenic lines against *Ornithogalum* mosaic virus	*Ornithogalum* plants showed to be susceptible to specific *Agrobacterium* strains. Transformation efficiency is dependent on the type of promoter used	**VAN EMMENES et al. (2008)**
Tricyrtis hirta Thunb.	Callus	*Agr*	EHA101	To establish a basic gene transfer protocol in Liliaceous species using *T. hirta* as a model plant	An efficient system for transgenic plant production was established	**ADACHI et al. (2005)**
Tricyrtis Wall. Sp. 'shinonome'	Callus	*Agr*	EHA101	To characterize transgenic *Tricyrtis*	Transgenic plants of *Tricyrtis sp* overexpressing GA2ox	**OTANI et al. (2013)**

				plants overexpressing gibberellin 2-oxidase (GA2ox)	exhibited dwarf phenotypes. The study indicated the possibility of molecular breeding for plant form modifications	
Tulipa gesneriana L.	Shoots	*Agr* and particle bombard-ment	A281, EHA101, C58C1, LBA4404	To compare efficiency of different systems of gene delivery and strains	*GUS* gene transfer was achieved and the basic protocol for transformation was established. Particle bombardment was the most effective	**WILMINK et al. (1992)**

Typha latifolia L.	Callus	*Agr*	EHA105, LBA4404	The aim was to develop a basic protocol for evaluating the effects of candidate genes for phytoremediation	Addition of acetosyringone in nutrient medium, wounding of explants and extended co-cultivation time significantly improved transformation	**NANDAKUMAR et al. (2004)**

**Agr = Agrobacterium*-mediated transformation

Micropropagation applications for ornamental geophytes are mostly aimed at mass propagation, germplasm conservation as well as forming a solid foundation for developing new cultivars through recombinant DNA techniques. These among others include developing cultivars for disease and viral resistance **(VAN EMMENES et al., 2008; KAMO et al., 2010)**, colour and scent enhancement or high throughput production of medically recognized phytochemicals **(COLLING et al., 2010)**, inflorescence yield, corolla size and flower longevity. For many geophytes, the most pertinent challenge is that of flowering time. The switch from the vegetative to flowering phase is caused by floral induction, which is dependent on endogenous signals such as age and environmental signals like day length and temperature **(NILSSON and WEIGEL, 1997)**. Most geophytes undergo a long juvenile phase of vegetative development which may last years before flowering **(LIN et al., 2003)**. Hence, shortening of juvenility in ornamental geophytes through genetic transformation can be of immense biotechnological interest and horticultural benefit.

The success of any genetic transformation strategy entirely depends on the regeneration capability of the explant. Although many regeneration protocols have long been established for ornamental monocotyledonous geophytes, the regeneration response has been mostly through direct organogenesis (see reviews by **HUSSEY, 1977 and ASCOUGH et al., 2009**). As an alternative to a regeneration pathway through direct organogenesis, somatic embryogenesis (organogenic or embryogenic callus production) has been used in micropropagation systems to assist genetic transformation. Therefore, regeneration response via somatic embryogenesis in monocotyledonous geophytes will

greatly facilitate their transformation. This highlights the need for somatic embryogenesis protocols to attain successful transformation.

2.2 Factors Affecting Genetic Transformation in Monocotyledonous Geophytes

2.2.1 Host and *Agrobacterium* factors

The molecular concepts underlying genetic transformation of plant cells by *Agrobacterium* are well known. Briefly, this involves the transfer of T-DNA; found within the tumor-inducing (*Ti*) plasmid from the *Agrobacterium* to the plant nuclear genome. This process is assisted by virulence genes carried by the *Ti* plasmid **(GELVIN, 2003)**. It is well recorded that the use of *Agrobacterium* for genetic transformation greatly facilitates stable integration of a single copy of the transgene in the plant genome with minimum or no re-arrangements of the foreign DNA structure. It is therefore known as a method with fewer complications such as transgene instability, gene silencing or co-suppression **(SOOD et al., 2011)** and this could greatly benefit transformation of monocotyledonous geophytes.

2.2.2 *Vir* inducers

During transformation, various virulent effector proteins (*Vir* proteins) are conveyed from the *Agrobacterium* to the host plant cells through the cell wall and the plasma membrane **(GELVIN, 2010)**. *Agrobacterium* possesses some sensors that enable it to recognize signals emitted by the host tissue and thus enable virulence in response to these signals

(LACROIX et al., 2011). Initially acetosyringone (3,5-dimethoxyacetophenone) was identified as one of the plant cell exudates (phenolic compound) shown to act as a *Vir* inducer with varying efficiencies depending on plant species **(PALMER et al., 2004)**. For instance, transformation frequency in *Trycirtis hirta*; an ornamental plant, was increased when acetosyringone (50 mg L^{-1}) was added to the co-cultivation medium **(ADACHI et al., 2005)**.

2.2.3 *Agrobacterium tumefaciens* strains

The type of strain used can affect transformation frequency. In *Iris germanica*, LBA4404 gave remarkably higher transformation rates than EHA105 **(JEKNIC et al., 1999)**. In *Agapanthus praecox*, the same LBA4404 was found to be more effective than EHA101 **(SUZUKI et al., 2001)**. The activity of LBA4404 is attributed to the super binary vector pTOK233, which has *VirB*, *VirC* and *VirG* genes derived from the 'supervirulent' Ti-plasmid; pTiBo542 **(SUZUKI et al., 2001)**.

2.2.4 Co-cultivation factors

Many geophytes are monocotyledonous plants that have previously been thought to be non-hosts of *Agrobacterium tumefaciens.* This is mainly due to the fact that monocotyledonous cells may sometimes produce unfavourable phenolic compounds in response to wounding **(PUDDEPHAT, 2003)**. However, **DANILOVA et al. (2006)** found that an extract of sterile tobacco leaves and stems increased maize transformation more effectively than acetosyringone. The stimulatory effects of tobacco were attributed to the

phenomenon that tobacco contains a wide range of favourable phenolic compounds, sugars and amino acids which induce *Vir* genes responsible for T-DNA transfer. Tobacco extract could equally be beneficial in transformation of ornamentals.

Co-cultivation period can also bring about the success or failure of transformation of a given plant. This period needs to be pre-determined to avoid a lower frequency of transformation or *Agrobacterium* overgrowth due to prolonged co-cultivation time. A co-cultivation period of 2-3 days provided best results in *Agapanthus praecox* **(SUZUKI et al., 2001)**, while in *Typha latifolia*, a three day co-cultivation resulted in the highest level of *GUS* expression **(NANDAKUMAR et al., 2004)**. Since T-DNA transfer from *Agrobacterium* into the plant genome occurs during the S-phase of the cell cycle **(VILLEMONT et al., 1997)**, it is essential to establish optimum co-culture conditions of explants and the *Agrobacterium* at the very beginning of the genetic transformation protocol.

2.2.5 Type of promoter fused to the coding region

Regulated promoters allow control of gene expression and facilitate the genetic improvement of important plants **(DAJMAL et al., 2010)**. Therefore, successful genetic modification of flowering bulbs with genes of interest requires the availability of promoters that can be characterized and expressed at functional levels **(KAMO et al., 2009)**. The most common and widely used, cauliflower mosaic virus (CaMV) 35S promoter, has resulted in lower levels of expression in some plants while in others, the results were satisfactory. This promoter was found to be the best for transformation of *Iris germanica*.

This outcome further confirmed that *Agrobacterium*-mediated transformation using CaMV35S can be applied to other important monocotyledonous ornamentals **(JEKNIC et al., 1999)**.

Another well-known promoter ubiquitin; is used in genetic engineering of monocotyledonous species since it promotes high levels of expression in most plant tissues **(KAMO et al., 2009)**. Two ubiquitin promoters were isolated from *Gladiolus* namely; GUBQ2 and GUBQ4. It was shown that levels of *GUS* expression were higher with the GUBQ4 promoter than with GUBQ2 **(KAMO et al., 2009)**. The GUBQ1 isolated from maize gave the highest level transient *GUS* expression in *Gladiolus* **(JOUNG and KAMO, 2006)**, while in *Ornithogalum* transformation this promoter was less efficient in expressing *GUS* when compared to the CaMV35S promoter **(DE VILLIERS et al., 2000)**. Identification and the use of efficient promoters in genetic modification of monocotyledonous geophytes must therefore be taken into consideration. Intensive research is needed to isolate and use promoters from each plant species to be transformed by their own active promoters. Research involving the discovery and characterization of new enhanced promoters with higher levels of constitutive expression is needed.

2.2.6 Explant age/source

The source of explant can determine the failure or success of transformation. The meristematic tissue whose cells receive the transgene must be able to recover from any shock inflicted by the transformation treatment and quickly regenerate into mature plants **(SOOD et al., 2011)**. Some reports have revealed that younger explants such as immature embryos can be transformed more efficiently than mature plants **(ZHAO et al., 2000)**. Shoot tips were used as explants in *Agrobacterium*-mediated transformation of *Gladiolus* **(BABU and CHAWLA, 2000)**. Leaf explants also resulted in successful transformation of *Hyacinthus orientalis* and *Narcissus tazzeta* **(LU et al., 2007; POPOWICH et al., 2007)**. The most common way of stable transformation of monocotyledonous geophytes has been through the use of callus **(Table 2.2)**. Thus far, somatic embryogenesis of ornamental geophytes has been achieved in several species including; *Crocus sativus*, *C. heuffelianus* **(BLAZQUEZ et al., 2009; DEMETER et al., 2010)**, *Fritillaria meleagris* **(PETRIC et al., 2011)** and *Merwilla plumbea* **(BASKARAN and VAN STADEN, 2012)**. This calls for more research in the development of somatic embryogenesis protocols in monocotyledonous geophytes to assist transformation.

2.2.7 Antibiotics for selection of transformed cells and plantlets

The most challenging aspect in the genetic manipulation of geophytes is establishing subsequent regeneration of plants after every transformation event. The post-agroinfection phase of explants involves their exposure to two forms of antibiotics; one for eliminating *Agrobacterium* (mostly cefotaxime) and the other for selection (mostly

kanamycin, hygromycin or phosphinothricin) of transformed plants **(MIKI and MCHUGH, 2004; HUSAINI et al., 2011)**. The concentration of these antibiotics has a significant impact on the regeneration and transformation efficiencies. The concentration of kanamycin used for selection of putative transformants varies with cultivars and explant types **(HUSAINI et al., 2011)**. Since selective agents such as kanamycin have been shown to interfere with regeneration, and that monocotyledonous geophytes are not natural hosts of the *Agrobacterium*, it is sometimes beneficial to involve a delay period of 2 to 10 days (pre-selection phase) before inoculating explants onto the selection medium; thus allowing the transformed explants to recover from the infection process and to express selectable marker genes **(ZHAO et al., 2004)**.

2.3 Past Efforts and Achievements in Transformation of Monocotyledonous Geophytes

Despite genetic engineering methods available, it has been observed that genes that could enhance the quality of ornamental geophytes are many but only a few have been characterized in ornamental geophytes. To date, only a few studies have involved the application of genetic transformation techniques other than reporter genes. *Lilium longiflorum* plants were transformed for resistance against cucumber mosaic virus (CMV) via particle bombardment **(LIPSKY et al., 2002)**. Phytoene synthase (PSY) is a regulatory enzyme for carotene biosynthesis and therefore important for colour formation. The PSY gene was used in the *Agrobacterium*-mediated transformation of *Narcissus tazzeta* var. *chinensis* **(LU et al., 2007)**. *Hyacinthus orientalis* cv. *Chine Pink* transformed with the thaumatin II gene showed a significant level of resistance to the pathogenic fungus;

Botrytis cinera **(POPOWICH et al., 2007)**. *Gladiolus* plants transformed with a defective replicase and protein subgroup II gene were found to be resistant to cucumber mosaic virus (CMV) **(KAMO et al., 2010)**, while **AZADI et al. (2011)** established that the integration of a defective CMV replicase gene (CMV2-GDD) resulted in virus resistant *Lilium* plants.

Since in ornamental floriculture, more emphasis is laid on flower quality and related characteristics (such as petal colour, size and scent), genetics in floral development has become an important discipline. Molecular genetic studies have identified many genes and other regulators that play important roles in floral development. These studies have yielded important insights into the control of flower development, thereby adding to the widely available genetic database for well-established models **(BUZGO et al., 2004)**. For instance, a flower regeneration system was set up for *Saussurea involucrata*. This was to facilitate basic biological studies of flower development by introducing heading-date 3a (*Hd3a)*; the gene responsible for early flower induction **(LI et al., 2011)**.

2.4 Current Trends and Other Applicable Methods

Most systems currently or previously used are *Agrobacterium*-mediated based methods of transformation. *Agrobacterium*-mediated transformation is a widely utilized method of gene delivery. It can assume many forms or systems as outlined below. These systems can be employed individually or concurrently. **Table 2.2** gives some of the examples where these systems are or have been employed.

2.4.1 Agrobacterial monolayer

Most approaches to improve bacterial penetration in monocotyledonous plants involve wounding before or during co-culturing. This is to facilitate transfer of *Agrobacterium* genes across the plant cell walls. However, these mechanical treatments may sometimes damage or deteriorate the physiological state of the explants to an extent that growth retardation and reduced regeneration capacity may result **(DANILOVA et al., 2006)**. The monolayer system uses a long co-cultivation period (about 15 to 20 days) of plant tissues and *Agrobacterium* to provide a high possibility of transformation, while at the same time not imposing adverse effects on tissue regeneration **(DANILOVA et al., 2009)**. To prepare a monolayer, the *Agrobacterium* is grown overnight and about 1 mL of the suspension is transferred and spread evenly on Petri dishes containing agar-solidified nutrient medium. Petri dishes are then left under laminar flow for 10-15 minutes for slight drying. Plant tissues are then inoculated over the bacterial monolayer and co-cultured **(DANILOVA et al., 2009)**. This system was successful for maize transformation **(DANILOVA et al., 2009)**. It was used in *Agrobacterium*-mediated transformation of *Dierama erectum*; a monocotyledonous ornamental geophyte and results have shown that it is one of the best gene delivery systems (see **Chapter 4**) and could be applied for most monocotyledonous geophytes.

2.4.2 Floral dip method

This is an in planta transformation procedure (non- tissue culture based) in which the basal medium containing *Agrobacterium* carrying constructs of interest, is pipetted into

open plant florets during anthesis. In monocotyledonous plants, best results are obtained when spikes have not yet emerged from the sheaths. Florets are then covered to create enough humidity and later uncovered and air dried. The mature T_1 seeds are then screened for transformation. This method has been applied for stable transformation of wheat **(ZALE et al., 2009)** and could be easily applied on flower buds of monocotyledonous geophytes.

2.4.3 Microparticle bombardment

In short, this involves the direct delivery of exogenous DNA into plant cells. The genetic material is precipitated onto micron-sized tungsten or gold particles. These are placed within a barrel designed to accelerate them to velocities needed to penetrate the cell wall **(TAYLOR and FAUQUET, 2002)**. The limiting factors in developing transgenic ornamental bulbs can be overcome by direct DNA transfer methods; thus by-passing the barriers imposed by *Agrobacterium*-host specificity and monocotyledonous plant cell constraints **(CHRISTOU, 1995)**. Some advantages offered by this system include; transformation of organized tissue, rapid discovery of transformed T_1 seeds, transformation of recalcitrant species and also offering the basis for studying many plant developmental processes **(CHRISTOU, 1992)**. This technique has been applied to obtain transgenic plants of tulip **(WILMINK et al., 1992)**, *Lilium longiflorum* and *Ornithogalum dubium* **(COHEN et al., 2004)**. Recently, a successful genetic transformation protocol for *Gladiolus*; a monocotyledonous flower bulb using particle bombardment has been reported **(KAMO et al., 2009)**.

2.5 Challenges Encountered in Transformation

Genetic transformation in monocotyledonous geophytes is impeded by availability of somatic embryogenesis protocols for specific plant species (see examples given in **Table 2.1**). The standardization of somatic embryogenesis does not only help maintain and enhance the multiplication of elite clones of interest for high productivity, but also for the establishment and utility of a given transformation protocol in genetic engineering **(KUMAR et al., 2006)**. Somatic embryogenesis has been considered as the basic tool for transformation studies especially in genetic transformation methodologies involving *Agrobacterium* and biolistics **(PARIMALAN et al., 2011)**. It may well be stated that this is a necessity in the genetic modification of ornamental geophytes. The long juvenility phase of these geophytes is the other factor prolonging their genetic modification, since *in situ* methods such as pollen transformation would have to be performed only after flowers have emerged.

2.6 Future Research and Other Gene Delivery Systems to be Utilized

2.6.1 Integration of new genes

Identification of new genes of interest together with the application of those that have proven successful in other plant species can be of immense horticultural benefit. For instance; the *MADS*-box genes which encode transcription factors involved in transition from vegetative to reproductive growth, determination of floral organ identity, senescence and many other developmental processes in plants **(BECKER and THEISSEN, 2003;**

HOENIKA et al., 2008) can be utilised. The ectopic expression of *OsMADS1* in transgenic tobacco plants resulted in early flowering plants **(CHUNG et al., 1994)**. Another *MADS*-box gene isolated from silver birch; *BpMADS4*, prevents normal senescence, winter dormancy in *Populus tremula* **(HOENIKA et al., 2008)** and promotes early flowering in apple **(FLACHOWSKY et al., 2007)**.

In recent years, the *FLOWERING LOCUS T* (FT) gene has been the most widely used and effective in promoting early flowering in various plants. Its homologous genes such as *PtFT1*, *CiFT*, *Hd3a* and *SFT* have recently been isolated from poplar, citrus, rice and tomato, respectively **(XU et al., 2012)**. The discovery of the *FT* gene raised interest in the study of *FT* genes in different species **(XU et al., 2012)**. *FT* homologous genes have been isolated and their roles have been studied extensively. For instance, it was found that under short day conditions in Kasalath (a rice cultivar), an ortholog of *FT; Hd3a* promotes early flowering **(KOJIMA et al., 2002)**. In another study **TAMAKI et al. (2007)** showed that the same *Hd3a* induces flowering in rice. Further investigations on the activity of these genes on ornamental geophytes, together with other programs aimed towards identification of beneficial genes are therefore valuable. Future research on these plants can also involve those aspects of genetic transformation which have not yet been explored in ornamental monocotyledonous geophytes. This includes utilization of the methods described below:

2.6.2 *Agrobacterium*-mediated gene transfer into seeds

Another approach that has recently received more attention is one which involves gene expression that does not require tissue culture; *Agrobacterium* gene transfer into seeds. It is considered to be a faster and less laborious approach of generating transgenic plants. To transform the seeds, the *Agrobacterium* containing a gene of interest is prepared. Dormant seeds are aseptically decontaminated, trimmed and co-cultivated with *A. tumefaciens*. This technique allows the *Agrobacterium* to penetrate intracellular spaces in the seed tissue and finally transform the embryo cells. Germinated seedlings are then transferred to soil for growth and further analysis are performed accordingly **(FURSOVA et al., 2012)**. Although there are no reports on the application of this technique in ornamental monocotyledonous geophytes, results obtained from transformation of *Brachypodium distachyon* (grass species), show that this system could be applied to other monocotyledonous species in future **(FURSOVA et al., 2012)**.

2.6.3 Sonication-assisted *Agrobacterium*-mediated transformation

Due to some transformation difficulties encountered in monocotyledonous species as mentioned earlier, the *Agrobacterium* may fail to reach the target cells. **TRICK and FINER (1997)** described sonication-assisted *Agrobacterium* transformation (SAAT) as a tool that allows for effective delivery of T-DNA from *Agrobacterium* to a large number of cells in the plant tissues. This includes diverse groups of plants; dicotyledons, monocotyledons and gymnosperms. The technique simply involves exposure of plant tissue to ultrasound for a short duration (few seconds) in the presence of *Agrobacterium*. It was found that

SAAT treatments produce fissures (micro-wounds) that assist the *Agrobacterium* to easily reach internal plant tissues thereby increasing chances for transformation events **(TRICK and FINER, 1997)**. In some studies, SAAT has proven to be effective even at low *Agrobacterium* optical density (OD_{600nm} 0.11) **(SANTARÉM et al., 1998)**. This technique has great potential to be applied in genetic modification of monocotyledonous geophytes.

2.6.4 *Agrobacterium*-mediated gene transfer into pollen

Stable transformation of some monocotyledonous plants has been achieved through another non-tissue culture based technique; pollen transformation. This involves production of transgenic plants by inoculating florets with *Agrobacterium* at or near anthesis. This procedure leads to production of embryos with enhanced resistance to antibiotics during the selection phase for transformants. It has been successful in cereal crops such as barley, maize and wheat **(LANGRIDGE et al., 1992)**.

2.7 Recent Promising Prospects for Genetic Modification in Ornamental Geophytes

Successful horticultural trade with ornamental geophytes can be improved through application of multidimensional approaches towards the genetic enhancement of existing crops and further development of new ones **(KAMENETSKY, 2011)**. Genetic transformation has become an effective tool and thus future research on ornamental geophytes will utilize this technique. As more genes are being isolated, more options are becoming available for the application of genetic modification in ornamental geophytes.

KRENS and KAMO (2013) have listed more than 30 genes isolated and characterized from geophytes themselves. Some of these genes are involved in virus-, fungi- and insect-defenses as well as carotenoid biosynthesis. The majority of genes such as the MADS box genes that play a role in flower and color development, have also been isolated and successfully characterized. Strategies for flower architecture, color, scent modification and control of florigenesis via genetic engineering with special attention on metabolic engineering of the flavonoid pathway can also be applied **(TANAKA et al., 2005)**. Important traits such as vase life are also open to genetic modification. Ethylene is involved in senescence in many flowers and vase life can be lengthened by blocking ethylene biosynthesis **(SAVIN et al., 1995)**. Another important aspect that has shown great potential lately is the manipulation of biochemical pathways leading to production of highly valued plant secondary metabolites. Recent developments in the *Agrobacterium*-mediated transformation in monocotyledonous species can benefit from greater involvement in genetic modification of ornamental geophytes. **ZHANG et al. (2013)** have discovered that weakening defense responses in plants (related to the recognition of pathogen-associated molecular patterns and induced expression of genes linked to pathogenesis in monocotyledonous plants), can enhance *Agrobacterium*-mediated transformation.

2.8 Summary

The ability to successfully establish micropropagation protocols (especially somatic embryogenesis) for monocotyledonous geophytes is critical as it serves as a pre-requisite to genetic transformation. With sustained interest in ornamental plants and availability of techniques such as *Agrobacterium*-based systems of gene delivery and biolistic methods, together with continuous research in gene isolation, there is a promising future for the success of genetic modification of monocotyledonous ornamental geophytes. This review presents an outline of some of the examples whereby most of the monocotyledonous species listed have been successfully transformed through *Agrobacterium*-based methods. However, most of the protocols given are either basic or done to induce disease resistance **(Table 2.2)**.

In view of recent promising prospects, the floriculture industry still has a potential to offer more satisfactory products to consumers. While these facts remain true, identification of genes and promoters that can be expressed at a functional level for monocotyledonous geophytes are still challenging. The knowledge of monocotyledonous host cell cycles, establishment of somatic embryogenesis protocols and their correlation with mechanisms for T-DNA transfer needs to be explored in depth.

CHAPTER 3
CALLUS INDUCTION AND ORGANOGENESIS

3.1 Introduction

The most commonly used micropropagation techniques are direct shoot organogenesis and somatic embryogenesis. The latter is more desirable for plant genetic transformation. Somatic embryogenesis is the ability of somatic cells to form embryos by a process resembling zygotic embryogenesis. The process leads to the formation of a bipolar structure with root and shoot axes and a well-functioning vascular system. In monocotyledonous plants, somatic embryo stages such as the globular, coleoptilar and scutellar can be observed **(THORPE and STASOLLA, 2001)**.

Under appropriate conditions, some somatic cells are capable of undergoing somatic embryogenesis while some may develop root or shoot structures only (organogenesis). In organogenic callus production; shoot buds usually develop from nodular structures. This ability to regenerate plantlets from callus in some Iridaceae species was first reported by **HUSSEY (1975)**. Not only does this unique developmental stage provide a basis for mass propagation but it also forms a foundation for experimental models aimed at understanding the molecular basis of development in plants **(FEHÉR et al., 2003)**.

In most cases, pollination and fertilization are known as prerequisites for embryo initiation in seed development. In somatic embryogenesis however, there are major differences. Unlike in seeds, there is neither endosperm differentiation, embryo desiccation nor dormancy processes **(FEHÉR et al., 2003)**. Induction of somatic embryogenesis can be

triggered by many factors including; stress, plant growth regulators, high temperature, elevated sucrose concentrations, and various concentrations of mineral elements in the growth medium **(GAJ, 2004)**.

The establishment of an efficient protocol for regeneration and genetic transformation is essential for the incorporation of useful traits in horticultural plants. One of the critical steps in transformation systems is the establishment of optimal conditions for efficient T-DNA delivery into the target tissue. This part of the study was aimed at developing a protocol for callus production so as to facilitate rapid regeneration of *Dierama erectum* transformants.

3.2 Materials and Methods

3.2.1 Culture media and growth conditions

Unless stated otherwise, the basal media consisted of Murashige and Skoog (MS) **(MURASHIGE and SKOOG, 1962)** salts supplemented with 30 g L^{-1} sucrose and solidified with 8 g L^{-1} agar. The pH of all media was adjusted to 5.8, and different concentrations of PGRs were added before autoclaving for 20 min at 121 ˚C. Cultures were maintained in a growth chamber at 25 ˚C under a 16-h light period, using a total irradiance of 82 µmol $m^{-2}s^{-1}$ provided by cool white fluorescent lamps (110 W, Phillips, USA, 75% total wattage).

3.2.2 Callus induction and regeneration

Seeds of *D. erectum* were washed in running tap water, surface sterilised with 0.1% (w/v) mercuric chloride for 10 min under agitation, and then rinsed five times with sterile distilled water. Disinfected seeds were germinated on 1/10th strength MS medium without sucrose for 12 days. Various treatments were employed to induce embryogenic callus from seedling-derived shoot apical meristems (SAMs). Firstly, 1-naphthaleneacetic acid (NAA) and 2,4-dichlorophenoxyacetic acid (2,4-D) at concentrations of 0, 0.5, 1.0, 2.0 and 2.5 mg L^{-1} were added to a full strength MS medium. Thereafter different combinations of auxins (NAA, Picloram and 2,4-D) at 1.0 mg L^{-1} and cytokinins (6-benzyladenine (BA), *meta*-toplin (*m*T) and thidiazuron (TDZ)) at 0.1 mg L^{-1} together with different sucrose

levels (30, 35 and 40 mg L^{-1}), were tested for their effect on callus induction and regeneration.

3.2.3 Microscopic evaluation of callus and regenerated shoots

The images were recorded using a stereomicroscope (Leica MZ16, Switzerland) fitted with an image capturing system (Leica DFC450C, Germany) compatible with LAS Version 4 software. Fresh plant materials were viewed directly without any pre-treatment.

3.2.4 Rooting and acclimatization

Shoots obtained from the medium containing 1.0 mg L^{-1} NAA, 0.1 mg L^{-1} *m*T and 35 g L^{-1} sucrose were carefully separated and placed on MS fortified with different IBA concentrations (0, 0.2 and 0.4 mg L^{-1}). Following a 4 week period, plants were transferred to potting soil and placed in the mist house for 2 weeks after which they were monitored in the greenhouse for another 4 week period and the percentage survival was then recorded.

3.2.5 Data analysis

The experiments were repeated at least twice using groups of 18 or 24 explants. Data were subjected to analysis of variance (ANOVA), and comparisons of means were carried out with Duncan's Multiple Range Test at 0.05% significance level.

3.3 Results and Discussion

3.3.1 Callus induction and regeneration

Table 3.1 shows the effects of various NAA and 2,4-D concentrations on the number of shoot apical meristems forming organogenic callus, and the number of shoots produced per explant. It is apparent that *D. erectum* was capable of producing callus with NAA concentrations between 0.5 and 2.0 mg L^{-1}. The highest callus formation (2.50 ± 0.34 explants forming callus) was obtained when the MS was supplemented with 1.0 mg L^{-1} NAA. Increasing the NAA concentration beyond 2.0 mg L^{-1} resulted in less callus production associated with lower shoot regeneration from the callus. NAA concentrations varying from 1 to 10 mg L^{-1} were found to be necessary for callus induction in *Gladiolus* **(KAMO et al., 2010)**. This is consistent with the findings in this experiment. On the other hand, 2,4-D which is often used for callus induction, resulted in no callus induction in this experiment. This indicates that its application on *D. erectum* had detrimental effects on this species.

An investigation to obtain the best culture media combination for embryogenic callus induction was conducted by employing various combinations of PGRs together with different sucrose concentrations. The attempt to obtain embryogenic calli from shoot apical meristems of *D. erectum* was met with limited success since the callus obtained was more of an organogenic type instead of the embryogenic type **(Figure 3.1A)**. Macroscopic observations revealed that the established callus lacked the globular, coleoptilar and scutellar stages normally observed with somatic embryogenesis **(Figure**

3.1). Some callus obtained with 2.0 mg L^{-1} NAA was hard, green and non-organogenic **(Figure 3.1B)**.

When comparing results in **Tables 3.1** and **3.2**, it is clear that NAA alone induced more callus than in combination with BA, *m*T and sucrose. Callus induction was later followed by multiple shoot production from the same callus. The shoots did not have any roots until they were transferred to a PGR-free medium **(Figure 3.1D)**. The organogenic callus obtained from this experiment was subsequently used in the *Agrobacterium*-mediated transformation experiments **(Chapter 4)**.

Table 3.1 Effect of NAA and 2,4-D on callus induction and shoot production of *Dierama erectum*

Auxin concentration (mg L^{-1})		No. of SAMs forming callus at 8 weeks	No. of shoots per explant at 8 weeks
Control	0.0	0.00 ± 0.000^{c}	1.00 ± 0.00^{bc}
NAA	0.5	2.33 ± 0.210^{a}	2.94 ± 0.29^{a}
	1.0	2.50 ± 0.340^{a}	1.50 ± 0.19^{b}
	2.0	1.83 ± 0.600^{ab}	0.72 ± 0.21^{c}
	2.5	0.83 ± 0.540^{bc}	0.61 ± 0.12^{c}
2,4-D	0.5	0.00 ± 0.000^{c}	1.61 ± 0.20^{b}
	1.0	0.00 ± 0.000^{c}	1.33 ± 0.32^{b}
	2.0	0.00 ± 0.000^{c}	1.44 ± 0.26^{b}
	2.5	0.00 ± 0.000^{c}	0.94 ± 0.0056^{c}

Values in columns with different letter(s) indicate significant differences between treatments ($P \leq 0.05$, n = 18) based on Duncan's Multiple Range Test

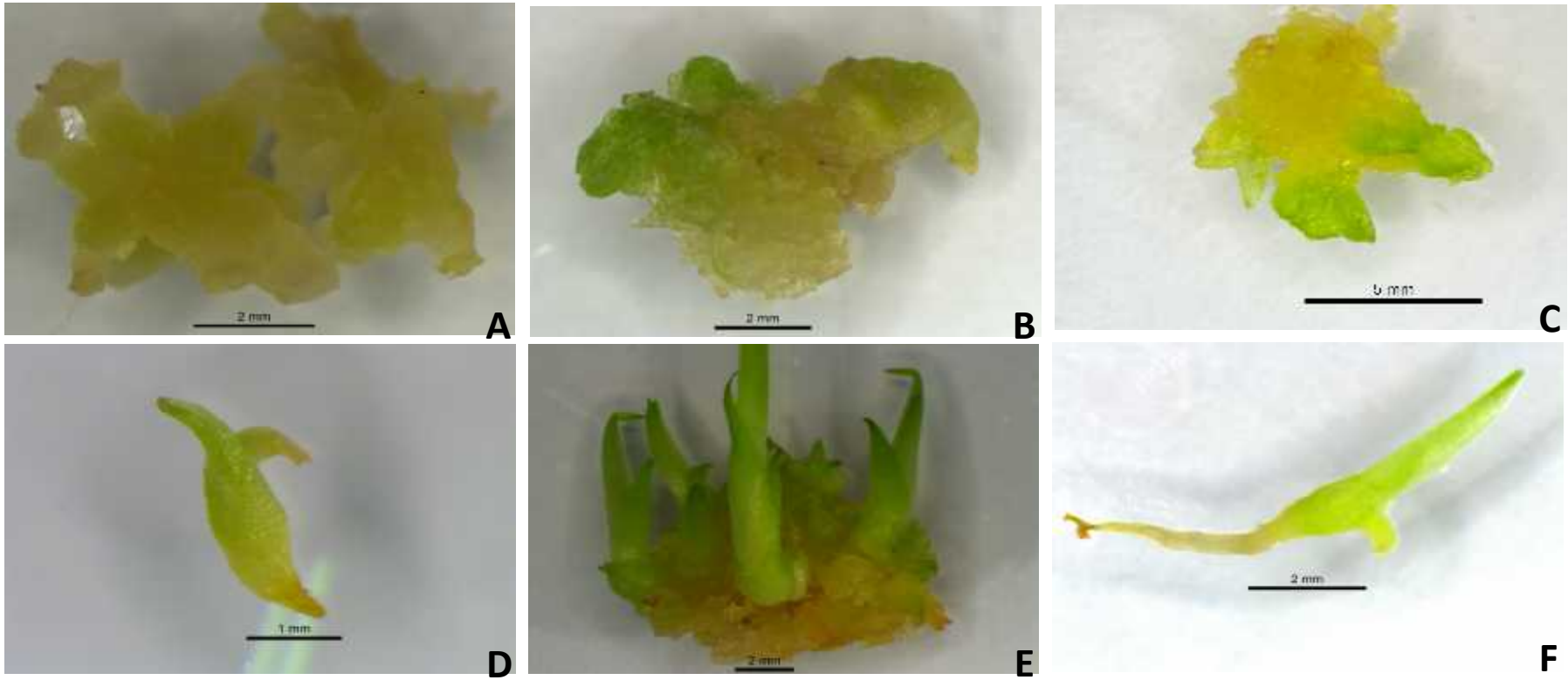

Figure 3.1: Effects of NAA on callus formation and subsequent shoot production of *Dierama erectum*. (**A**) Friable organogenic callus showing shoot primordia (1.0 mg L^{-1} NAA). (**B**) Non-organogenic callus (2.5 mg L^{-1} NAA). (**C**) Shoots developing from friable callus (1.0 mg L^{-1} NAA). (**D**) An individual shoot before transfer to PGR-free medium. (**E**) Shoot development after 12 weeks in culture (1.0 mg L^{-1} NAA + 0.1 mg L^{-1} + *m*T + 35 g L^{-1}). (**F**) Shoot and root development after transfer to PGR-free medium.

Table 3.2 shows results on the effects of auxin and cytokinin combinations and elevated sucrose concentrations on callus and shoots production. In general, the mean number of explants producing callus from all the combinations was very low when compared to NAA applications alone **(Table 3.1)**; even at the same 8 weeks period of culture (in fact, NAA application alone was 95% better). Instead, shoot multiplication was promoted especially when the cultures were left in the same medium for 12 weeks.

Callus induction was further deterred with combinations of NAA and cytokinins (BA and *m*T) even where the ratio of auxin to cytokinin was high rather than when NAA was applied alone. This is contrary to what **DEMETER et al. (2010)** reported; globular stage embryos were formed on a high auxin to cytokinin ratio (10 mg L^{-1} NAA and 1 mg L^{-1} BA). **KOETLE (2009)** obtained both organogenic callus and multiple shoots with NAA: BA ratios of 1: 2, 1: 4 and 1: 5. The NAA: BA ratio applied in this experiment was 1: 0.1, however no globular stage embryos were noted. Moreover, a clear understanding of the concepts behind embryogenic callus induction could be better understood through the adoption of molecular based studies. Another important aspect of these results lie in the fact that a combination of 1.0 mg L^{-1} 2,4-D and 0.1 mg L^{-1} *m*T encouraged shoot formation instead of callus. This might indicate some kind of superiority of this topolin in shoot regeneration **(PALAVAN-ÜNSAL et al., 2002)** even when a high concentration of 2,4-D is combined with it. Although a combination of 1.0 mg L^{-1} Picloram, 0.1 mg L^{-1} TDZ and 35 g L^{-1} sucrose had most explants producing callus (0.42 ± 0.1000), it was still significantly lower (by 80%) than when NAA was applied alone.

Table 3.2 Effect of auxin/cytokinin combinations and varied sucrose levels on callus initiation in *Dierama erectum*

Combined auxins and cytokinins	Mean no. of explants forming callus at 8 weeks	Mean no. of shoots/explant at 8 weeks	Mean no. of shoots/explant at 12 weeks	Mean no. of browned explants
Control	0.00 ± 0.00^{d}	1.00 ± 0.00^{bc}	1.00 ± 0.00^{c}	0.00 ± 0.00^{c}
1.0 mg L^{-1} NAA + 0.1 mg L^{-1} BA + 30 g L^{-1} sucrose	0.21 ± 0.0085^{bc}	2.38 ± 0.45^{a}	5.42 ± 0.41^{b}	0.21 ± 0.0085^{bc}
1.0 mg L^{-1} NAA + 0.1 mg L^{-1} BA + 35 g L^{-1} sucrose	0.00 ± 0.00^{d}	2.76 ± 0.25^{a}	6.34 ± 0.21^{ab}	0.27 ± 0.066^{bc}
1.0 mg L^{-1} NAA + 0.1 mg L^{-1} BA + 40 g L^{-1} sucrose	0.00 ± 0.00^{d}	1.27 ± 0.28^{b}	5.62 ± 0.37^{b}	0.00 ± 0.00^{c}
1.0 mg L^{-1} NAA + 0.1 mg L^{-1} *m*T + 30 g L^{-1} sucrose	0.00 ± 0.00^{d}	2.24 ± 0.39^{a}	6.14 ± 0.51^{ab}	0.31 ± 0.061bc
1.0 mg L^{-1} NAA + 0.1 mg L^{-1} *m*T + 35 g L^{-1} sucrose	0.29 ± 0.0095^{a}	2.71 ± 0.47^{a}	6.33 ± 0.47^{ab}	0.21 ± 0.0085^{bc}

1.0 mg L^{-1} NAA+ 0.1 mg L^{-1} *m*T + 40 g L^{-1} sucrose	0.14 ± 0.0027^{cd}	0.96 ± 0.39^{bc}	5.54 ± 0.38^{b}	0.42 ± 0.1000^{b}
1.0 mg L^{-1} 2,4-D + 0.1 mg L^{-1} *m*T + 30 g L^{-1} sucrose	0.00 ±0.00^{d}	1.81 ± 0.31^{ab}	6.56 ± 0.71^{ab}	0.22 ± 0.0076^{bc}
1.0 mg L^{-1} 2,4-D + 0.1 mg L^{-1} *m*T + 35 g L^{-1} sucrose	0.0042± 0.0042^{cd}	2.33 ± 0.41^{a}	7.25 ± 0.36^{a}	0.13 ± 0.0069^{c}
1.0 mg L^{-1} 2,4-D + 0.1 mg L^{-1}*m*T + 40 g L^{-1} sucrose	0.00 ± 0.00^{d}	1.88 ± 0.29^{ab}	6.25 ± 0.52^{ab}	0.13 ± 0.0069^{c}
1.0 mg L^{-1} Picloram + 0.1 mg L^{-1} TDZ + 30 g L^{-1} sucrose	0.00 ± 0.00^{d}	0.00 ± 0.00^{c}	0.00 ± 0.00^{c}	0.94 ± 0.0062^{a}
1.0 mg L^{-1} Picloram + 0.1 mg L^{-1} TDZ + 35 g L^{-1} sucrose	0.42 ± 0.1000^{a}	0.00 ± 0.00^{c}	0.00 ± 0.00^{c}	0.67 ± 0.0098^{a}
1.0 mg L^{-1} Picloram + 0.1 mg L^{-1} TDZ + 40 g L^{-1} sucrose	0.00 ± 0.00^{d}	0.00 ± 0.00^{c}	0.00 ± 0.00^{c}	0.78 ± 0.0034^{a}

Values in columns with different letter(s) indicate significant differences among treatments ($P \leq 0.05$, n = 24) based on Duncan's Multiple Range Test.

Sucrose is used in the medium as an energy source and acts as an osmotic regulator. An elevated sucrose level is sometimes considered as an important factor in callus induction **(ARZATE-FERNÁNDEZ et al., 1997; MENDOZA and KAEPPLER, 2002)**. However, in this study the opposite was observed. The increased sucrose concentrations did not assist in callus induction across all combined cytokinins and auxins used in this experiment.

3.3.2 Rooting and acclimatization

The data presented in **Figures 3.2** and **3.3** shows the effect of IBA on rooting and shooting in *D. erectum*. Both roots and shoots increased in the presence of 0.2 mg L^{-1} and 0.4 mg L^{-1} IBA and were significantly higher than the control. Continued shoot production in the rooting media might be an indication that *m*T might have had a carry-over effect on the regenerants since these were obtained from the medium containing 1.0 mg L^{-1} NAA, 0.1 mg L^{-1} *m*T and 35 g L^{-1} sucrose. Increasing the auxin concentration from 0.2 mg L^{-1} to 0.4 mg L^{-1} did not significantly increase either the number of roots or shoots. The same concentration (0.2 mg L^{-1}) led to successful rooting on the direct adventitious shoot regenerants of *D. erectum* **(KOETLE et al., 2010)**, indicating that the same rooting medium can be used in both direct and indirect regeneration protocols of this plant.

Rooted plants were transferred to potting soil, placed in the mist house for 2 weeks, after which they were transferred to the greenhouse. Only 62% of the plants survived. This was partly due to root-rot and the high moisture content in the mist house.

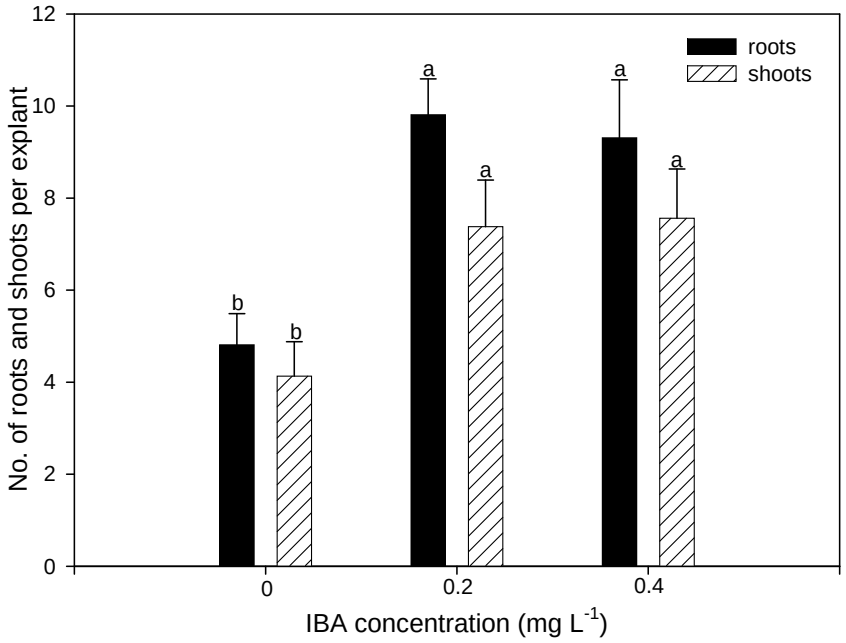

Figure 3.2 Effects of IBA on rooting and shoot production in *D. erectum.* Bars with different letter(s) indicate significant differences between treatments (P ≤0.05, n = 24) based on Duncan's Multiple Range Test

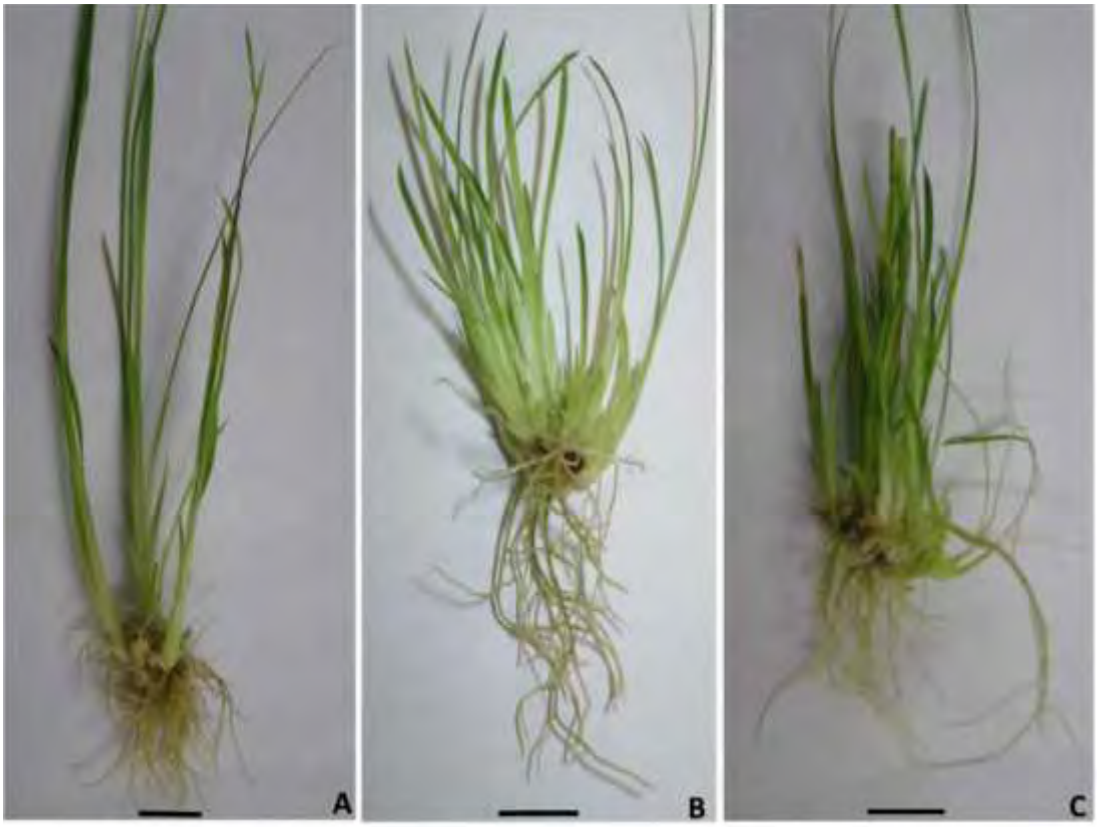

Figure 3.3 Effects of IBA on rooting and shoot production in *D. erectum*. **A** = 0 mg L^{-1} IBA, **B** = 0.2 mg L^{-1} IBA, **C** = 0.4 mg L^{-1} IBA, Bar = 1 cm

3.4 Summary

This study aimed at developing a callus induction protocol that would aid in regeneration of *D. erectum* transformants. Although it was expected that embryogenic callus would be obtained from shoot apical meristems, the experiments could only result in organogenic callus production. This calls for more investigations to further understand the requirements of successful embryogenic callus induction in *D. erectum*. In short, the experiments here reported:

- Establishment of an organogenic callus induction protocol that has potential for use in multiplication of transformed *D. erectum* regenerants. ;
- Identification of the best medium for callus induction (1.0 mg L^{-1} NAA);
- That increased sucrose concentration in *D. erectum* growth medium is not necessary for callus initiation; and
- That successful rooting, further shoot production and development as well as acclimatization of callus-derived plants were achieved with IBA.

CHAPTER 4
AGROBACTERIUM-MEDIATED TRANSFORMATION: OPTIMIZING FACTORS AFFECTING *GUS* GENE EXPRESSION

4.1 Introduction

Agrobacterium tumefaciens genetically transforms a plant by transferring T-DNA into the plant genome. The T-DNA is accompanied by various virulence (*Vir*) proteins which aid in its transfer, nuclear targeting and integration **(GELVIN, 2000)**. The expression of genes in the *Vir* region is induced by phenolics such as acetosyringone that are mainly found in the wound exudates **(ZUPAN et al., 2000)**. For this reason, acetosyringone is usually added to the growth medium to increase chances of *A. tumefaciens* transformation; especially of monocotyledonous plants where it is not synthesized naturally.

Dierama erectum Hilliard (Iridaceae) is a geophyte grown for medicinal and ornamental purposes. One outstanding feature of this species is the large-sized, magenta-pink flowers (see **Figure 4.1**); allowing for its development as an ornamental plant. Challenges facing *Agrobacterium*-mediated transformation of monocotyledonous geophytes such as *Dierama* are well known **(Chapter 2)**. Despite this, many related species have reportedly been successfully transformed via *Agrobacterium*-based systems of gene delivery. For instance, cormels and shoot tips of a close relative; *Gladiolus* were transformed **(BABU and CHAWLA, 2000; KAMO et al., 2010)**. Other examples include; *Agapanthus praecox* **(SUZUKI et al., 2001)**, *Narcissus tazzeta* **(LU et al., 2007)** and *Allium sativum* **(KONDO**

et al., 2000). This part of the study investigated the fundamental factors contributing to successful basic *Agrobacterium*-mediated genetic transformation in *D. erectum*.

Figure 4.1 Morphology of *Dierama erectum* (foliage and flowers)

4.2 Materials and Methods

4.2.1 Plant material and explant selection

Mature seeds were collected from the wild growing plant population at Mt. Gilboa, KwaZulu-Natal (S 29˚15.873, E 30˚ 29.743 ± 5 m). The seeds were washed in running tap water and dried at room temperature. They were disinfected with 70% (v/v) ethanol for 8 min followed by 0.1% (w/v) mercuric chloride and 3 rinses in sterile distilled water. Seeds were germinated aseptically on 1/10th strength Murashige and Skoog medium at 25 ˚C and 16 h light. The organogenic callus was obtained by following callus induction experiments (see **Chapter 3**). The embryonic shoot apical meristems (ESAMs), hypocotyls were derived from the seedlings and used as explants for subsequent genetic transformation experiments.

4.2.2 Sensitivity test of explants to kanamycin and cefotaxime

Since antibiotics have a great effect on regeneration of explants, it is essential that prior to genetic transformation, the amount of antibiotics (in this case kanamycin and cefotaxime) that may inhibit shoot and root growth be determined. The non-transformed embryonic shoot apical meristems (ESAMs) were placed on shoot multiplication medium **(KOETLE et al., 2010)** fortified with 0.23 mg L^{-1} benzyladenine (BA) and various concentrations of cefotaxime and kanamycin (0, 25, 50, 100, 200 and 400 mg L^{-1}). The kanamycin concentration (100 mg L^{-1}) that was found to kill all explants was used for selection of putative transformants in the subsequent experiments (**Table 4.1**). For

determining the concentration of cefotaxime to be used in these experiments; ESAMs were inoculated in an overnight *Agrobacterium* suspension and placed on the same shoot multiplication medium supplemented with different cefotaxime concentrations (0, 25, 50, 100, 200 and 400 mg L^{-1}). The cefotaxime concentration that eliminated all the bacteria while maintaining regeneration was 100 mg L^{-1}. There was no bacterial growth noted around the edges of explants in treatment. This concentration was used in the experiments that followed.

Table 4.1 Effect of antibiotics on embryogenic shoot apical meristem survival of *Dierama erectum*

Antibiotic concentration (mg L^{-1})		Growth parameters		
Cefotaxime	Kanamycin	No.of regenerating shoots	No. of roots	Length of longest root (mm)
0	---	1.20 ± 0.13^{a}	2.10 ± 0.35^{a}	25.30 ± 1.55^{a}
25	---	1.20 ± 0.13^{a}	1.30 ± 0.15^{b}	25.20 ± 2.35^{a}
50	---	1.20 ± 0.13^{a}	1.00 ± 0.00^{bc}	16.60 ± 1.05^{b}
100	---	1.00 ± 0.00^{ab}	1.00 ± 0.00^{bc}	18.70 ± 1.86^{b}
200	---	0.80 ± 0.13^{b}	0.70 ± 0.21^{cd}	3.30 ± 0.97^{c}
400	---	0.70 ± 0.15^{b}	0.70 ± 0.30^{cd}	0.60 ± 0.31^{c}
---	25	0.80 ± 0.13^{b}	0.60 ± 0.27^{cd}	0.50 ± 0.32^{c}
---	50	0.20 ± 0.13^{c}	0.40 ± 0.16^{de}	0.45 ± 0.29^{c}
---	100	0.00 ± 0.00^{c}	0.00 ± 0.00^{e}	0.00 ± 0.00^{c}

---	200	0.00 ± 0.00^{c}	0.00 ± 0.00^{e}	0.00 ± 0.00^{c}
---	400	0.00 ± 0.00^{c}	0.00 ± 0.00^{e}	0.00 ± 0.00^{c}

In each column, values with different letter(s) indicate significant differences among treatments ($P \leq 0.05$, n = 25) based on Duncan's Multiple Range Test

4.2.3 *Agrobacterium tumefaciens*-mediated transformation

Various experiments investigating the effects of explant types (ESAMs, hypocotyls and organogenic callus), co-cultivation time (0, 1, 2, 3, 4 and 5 days), acetosyringone concentration (0, 25, 50, 100 and 200 mg L^{-1}), *Agrobacterium* concentration (OD_{600} of 0.0, 0.2 0.4, 0.8, 1.6 and 2.0), and different systems of gene delivery (Agrobacterial monolayer, Agrobacterial suspension and sonication-assisted *Agrobacterium*-mediated transformation (SAAT)) were conducted. For all experiments, the *Agrobacterium* strain LBA4404 harbouring the binary plasmid vector pCAMBIA1301, with the T-DNA region consisting of *GUS* gene driven by the Cauliflower Mosaic Virus 35S (CaMV35S) promoter, was grown overnight (up to mid-log phase) in Luria Broth (LB) medium consisting of 0.1 mg mL^{-1} kanamycin and 0.15 mg mL^{-1} rifampicin. It was pelleted at 5000 × *g* for 20 min, washed in antibiotic free LB medium, re-pelleted and re-suspended in the same fresh LB medium. Explants were co-infected in LB containing the *Agrobacterium* for 30 min before transfer to co-cultivation medium (MS + 0.23 mg L^{-1} BA). After a pre-determined co-cultivation period, explants were washed in 350 mg L^{-1} cefotaxime for 10 min and blotted on a sterile filter paper to remove excess bacteria. Explants were then inoculated onto the pre-selection phase medium (MS + 0.23 mg L^{-1} BA + 50 mg L^{-1}

cefotaxime). After 7 days of explant recovery, they were washed again in 350 mg L^{-1} cefotaxime and transferred to the selection medium ((MS + 0.23 mg L^{-1} BA + 100 mg L^{-1} cefotaxime + 100 mg L^{-1} kanamycin) for selection of putative transformants. Where applicable, the rooting medium (MS + 0.20 mg L^{-1} IBA + 100 mg L^{-1} cefotaxime + 100 mg L^{-1} kanamycin) was used.

4.2.3.1 Effect of explant type on efficiency of GUS gene expression

Different protocols were followed to assess which explant source is suitable for efficient transformation. Seeds were aseptically germinated and once the ESAMs emerged, they were excised and inoculated in MS medium fortified with 1.0 mg L^{-1} 1-naphthaleneacetic acid (NAA) to obtain organogenic callus (see **Chapter 3**). Some of the ESAMs were reserved for use in transformation, while some seedlings were left to grow for about 2 more weeks so that the hypocotyl explants could be isolated. All explants (callus, ESAMs and hypocotyls) were inoculated in an agrobacterial suspension and placed on co-cultivation medium (MS + 0.23 mg L^{-1} BA) until the bacteria was visible around the explants (after 3 days). After co-cultivation, explants were washed, transferred to pre-selection and selection media. The efficiency of *GUS* expression was determined thereafter.

4.2.3.2 Effect of gene delivery systems on efficiency of GUS gene expression

Given that SAAT involves wounding of ESAMs through sonication; a sensitivity test of explants to sonication (time of explant exposure to sonication) was done. The ESAMs

were aseptically inoculated in 20 mL sterile distilled water and put on a sonicator (JULABO LABOTECHNIK GMBH, West Germany) operating at a maximum frequency of 35 kHz for 0, 10, 20, 30, 40, 50 and 60 s (as illustrated in **Figure 4.2**) to determine the best duration for wounding of explants without detrimental effects to the tissue. Explants were then placed on MS medium containing 0.23 mg L^{-1} BA. After 10 days, the percentage number of surviving regenerating explants was recorded. This preliminary experiment revealed that explants could not be exposed to sonication beyond 30 s (**Figure 4.3**) since this would deter their regeneration ability. Therefore, for all experiments involving SAAT, explants were sonicated for 30 s.

Explant tissues were viewed under the scanning electron microscope (SEM) to further investigate the effect of sonication on meristematic cells. To achieve this, ESAMs were suspended in distilled water contained in a 50 mL conical flask and left on a sonicotor for 30 s. Control explants were only immersed in distilled water. Explants were then prepared for viewing on (SEM) using the procedure outlined in **Appendix 4.1**. Samples were viewed with the SEM (ZEISS EVO/LS15) fitted to a secondary electron detector compatible with SmartSEM V05.04.02.00 computer software.

Figure 4.2 Apparatus for sonication-assisted *Agrobacterium*-mediated transformation. **A** = Conical flask containing agrobacterial suspension and ESAMs; **B** = Water level; **C** = Sonicator; **D** = Time adjustment knob

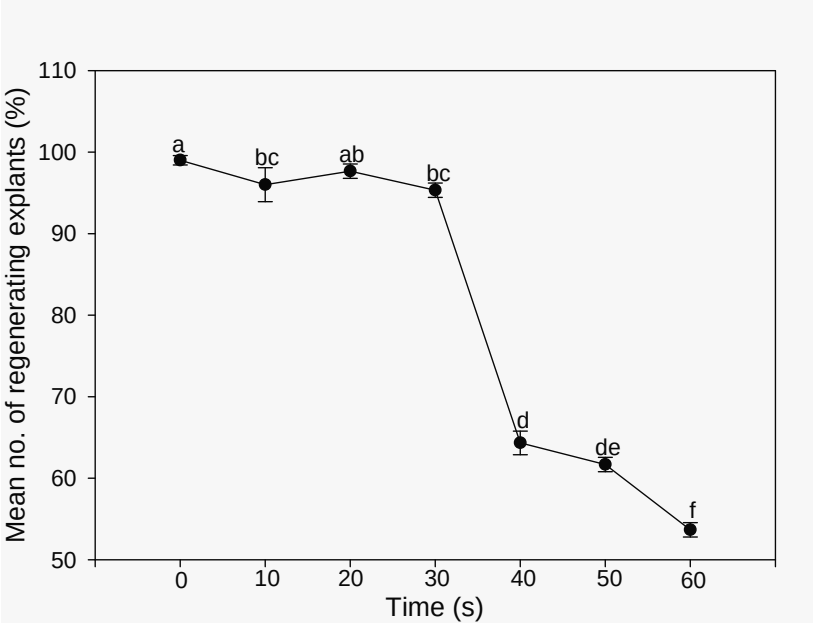

Figure 4.3 Effect of sonication on regeneration and survival of ESAMs. Means with different letters are significantly different from each other. Significant differences among treatments ($P \leq 0.05$, n = 42) were based on Duncan's Multiple Range Test

To investigate the effects of different systems of gene delivery on *GUS* expression, ESAMs were either inoculated in agrobacterial suspension (overnight culture) for 30 min without sonication, or in agrobacterial suspension and sonicated for 30 s or placed on an agrobacterial monolayer (preparation is described below). Plants that survived the selection phase (MS + 0.23 mg L^{-1} BA + 100 mg L^{-1} cefotaxime + 100 mg L^{-1} kanamycin), and grown for a further 4 weeks period, were evaluated for the efficiency of different systems on *GUS* expression.

4.2.3.2.1 Preparation of agrobacterial monolayer

A co-cultivation medium (MS + 0.23 mg L^{-1} BA) was prepared poured into the 12 cm diameter plastic Petri dishes. The agar was left to cool and 200 µL of an overnight *Agrobacterium* culture suspension was poured over the gel and spread evenly using a sterile glass “hockey stick”. The agar was left slightly opened until the liquid on the surface dried up (about 30 min). Embryonic shoot apical meristem explants were then inoculated and left on the co-cultivation medium for 21 days.

4.2.3.3 Effect of Agrobacterium concentration on efficiency of GUS expression

The *Agrobacterium* harbouring the *GUS* gene was cultured in liquid Luria Broth (LB) medium overnight. The bacterial concentration was adjusted accordingly such that optical density (OD_{600}) read 0 (no bacteria), 0.2, 0.4, 0.8 and 1.6. The ESAMs were inoculated in bacterial suspensions with different ODs, co-cultivated and placed on regeneration medium. The resulting regenerants were randomly selected for histochemical *GUS*

assay. Some were transferred to selection medium and after 4 weeks they were analyzed for *GUS* expression

4.2.3.4 Effect of acetosyringone concentrations on GUS expression

The *Agrobacterium* harbouring the *GUS* gene was cultured overnight in liquid Luria Broth (LB) medium supplemented with various acetosyringone concentrations (0, 25, 50, 100 and 200 mg L^{-1}). The ESAMs were inoculated in bacterial suspensions with different treatments, co-cultivated in the dark on MS medium supplemented with different acetosyringone concentrations. These were left in culture for 3 days. The resulting regenerants were randomly selected for histochemical *GUS* assay. Putative transformants were acquired from the selection medium and the analysis for *GUS* expression followed.

4.2.3.5 Effect of co-cultivation time on efficiency of GUS expression

The ESAMs were inoculated in *Agrobacterium* suspension (overnight culture). These were transferred to co-cultivation medium and left in culture for a number of days (0, 1, 2, 3, 4 and 5 days). After removal of excess bacteria with cefotaxime and blot drying on filter paper, plants were randomly selected for histochemical *GUS* assay. The remaining healthy plants were cultured on selection medium and analysis for *GUS* expression was done after 4 weeks.

4.2.4 Analysis of putative transformants

The method of histochemical *GUS* activity analysis was adopted from **JEFFERSON et al. (1987)** with some modifications. Resulting regenerants and/ or callus clusters, were incubated overnight at 37 ˚C in a buffer containing 0.5 mg mL^{-1} X-Gluc, 0.1 M sodium phosphate buffer (pH 7.0), 0.1 M potassium ferricyanide, 0.1 M ferrocyanide and 0.1% (v/v) Triton X-100, followed by treatment with 70% (v/v) ethanol for 24 h to remove chlorophyll. The efficiency of *GUS* expression (%) was calculated as number of plants expressing *GUS*/total number of plants evaluated × 100.

4.2.5 Proliferation, acclimatization and nomenclature of transformants

The regeneration and proliferation of plants were done using the optimized conditions above. Since the SAAT treatment gave more satisfactory results, this system was used for infection of ESAMs with the *Agrobacterium* to bulk up the plants that would be used for subsequent analysis. After DNA isolation from the leaves, plants were transferred to potting soil and left in the mist-house (26 ˚C) for a day, after which they were placed in the greenhouse (23-34 ˚C) in which they were watered daily. Plants were named in separate pots according to the gene delivery system they were generated from. The SAAT-derived plants were therefore named SAAT1, SAAT2, SAAT3 up to 17.

4.3 DNA Extraction, Quantification and Analysis

4.3.1 Plant genomic DNA isolation

Genomic DNA was isolated from leaves (0.25 g) of putatively transformed and untransformed plants (wild type) using the cetyltrimethylammonium bromide (CTAB) method described by **SAMBROOK et al. (1989)**. The leaves were frozen in liquid nitrogen in a sterilized mortar and ground to a fine powder. To the CTAB extraction solution (500 µL) were added; 3% (w/v) polyvinylpoly-pyrolindone (PVPP) and 10 µL of 7.5% (v/v) 2-mercaptoethanol (2-ME). The ground and frozen tissue was then added to 500 µL PVPP/2-ME/CTAB extraction solution (see **Appendix 4.2**). The mixture was then incubated for 30 min at 65 °C in a water bath with occasional mixing. The homogenate was mixed with an equal volume (500 µL) of 24:1 chloroform: iso-amyl alcohol by inversion followed by centrifugation at 7500 × *g* for 5 min in a microcentrifuge (HERMILE, Z160M). The upper aqueous phase (300 µL) was then recovered. The CTAB/NaCl solution (1/10th volume at 65 °C) was added to the recovered aqueous phase and mixed well by inversion. The mixture was then extracted with an equal volume (300 µL) of chloroform/iso-amyl alcohol. After mixing and centrifuging for 5 min at 7500 × *g*, the upper aqueous layer was obtained. One volume (1 mL) of CTAB precipitation solution was added to the recovered aqueous phase. An additional 30 min incubation at 65 °C was applied to allow for more DNA precipitation. The mixture was centrifuged for 5 min at 500 × *g* and the pellet was recovered by removing the supernatant. The pellet was suspended in 500 µL high-salt TE buffer (pH 8.0). The DNA was subsequently precipitated by adding 300 µL of ice-cold iso-propanol followed by mixing and centrifuging for 15 min at 7500 ×

g. The supernatant was decanted and the pellet was first washed in 80% ethanol followed by 100% ethanol. The air-dried pellet was suspended in 50 µl TE buffer and this DNA was stored at -20 ˚C for later use.

4.3.2 *Agrobacterium tumefaciens* plasmid isolation

Agrobacterium plasmid isolation was done according to **SAMBROOK and RUSSEL (2001)**.The *Agrobacterium* culture was inoculated in 10 mL Luria broth (LB) medium supplemented with 0.1 mg mL^{-1} kanamycin and 0.15 mg mL^{-1} rifampicin and shaken to early log phase (about 36 h). The 1 mL suspension culture was centrifuged at 12000 × *g* in a microcentrifuge for 10 min and the resultant pellet resuspended in 100 µL cell suspension solution (see **Appendix 4.2**). Added to this solution was 20 µl of a 20 mg mL^{-1} lysozyme. This was mixed gently and incubated at 37 °C for 15 min. Then 200 µl of cell lysis solution was added and mixed completely by repeated gentle inversion of the tube. The phenol solution (50 µL) equilibrated with 2 volumes (100 µL) of cell lysis solution was added and the mixture was vortexed. The neutralization solution (200 µL) was then added and the contents were mixed by repeated inversion of the tube. The mixture was then centrifuged at 12000 × *g* for 5 min, the acquired aqueous phase was transferred to the second eppendorf tube and to this was added 2.5 volumes of 95% ethanol and contents were placed on ice for 10 min. The DNA was spun down by centrifuging at 12000 × *g* for 5 min. The pellet was resuspended in 50 µL TE buffer and stored at -20 °C until required.

4.3.3 DNA quantification

Quantification was done for each sample using spectrophotometry at A_{260}, A_{280} and A_{320} nm absorbance using 250 × dilution (4 µL of DNA and 996 µL TE buffer). Ratio, concentration and purity were calculated using the following formulae:

Ratio = A_{260}/ A_{280}

Purity (%) = Ratio/1.8 × 100

Concentration (µg/µL) = (corrected A_{260} × ΔE × dilution)/1000, where:

Corrected A_{260} = A_{260} − A_{320}

ΔE = 50

Dilution factor = 250 ×

4.3.4 PCR analysis of transformants

Transgenic plant verification was performed with putative transformants and wild-type explants. Total genomic DNA was obtained from leaves and confirmation of the presence of the *GUS* gene in the plant genome was done using the following primer set **(MUHAMMAD et al., 2014)**:

GUS F: GTCGGCTTTCAGCTGTCTTT (T*m*; 58.77 °C)

GUS R: TGAGCGTCGCAGAACATTAC (T*m*; 58.65 °C)

The PCR reaction volume was 50 µL; of which 25 µL was PCR mastermix (Fermentas^{TM}), 1 µM forward primer, 1 µM reverse primer, and 0.02 µg μL^{-1} template DNA. Nuclease-free water was added to bring the final volume to 50 µL. The reaction was done using a

96 well Veriti ™ thermal cycler (Model #: 9902, Singapore) under the following cycling parameters; (1) initial denaturation for 1 min and 20 s at 98 ˚C, (2) 35 cycles of denaturation for 8 s at 98 ˚C, annealing for 30 s at 57 ˚C, extension for 50 s at 72 ˚C, and (3) final extension for 7 min at 72 ˚C. Following amplification, PCR products were analysed on agarose gel as described below. The expected product length of *GUS* was 595 bp.

4.3.5 Gel electophoresis

A 1% agarose gel was prepared and the heated mixture was allowed to cool to about 55 ˚C before pouring on the electrophoresis rig fitted with a comb. The buffer concentration (1 X TAE) in the gel was the same as that in the running buffer and the wells were oriented to the nearest negative electrode. A 5 µL of loading buffer (Glycerol-bromophenol blue) purchased from Fermentas^{TM}, was added to each 50 µL PCR sample and 20 µL of this was dispensed into a gel slot. In a separate well 20 µL (0.5 µg μL^{-1}) of DNA ladder mix (Thermoscientific GeneRuler 1 kb DNA ladder (250-10000 bp)) was added**.** Samples were electrophoresed at 7 volts/cm, until the bromophenol blue marker dye migrated to the end of the gel nearest to the positive electrode. The gel rig was disconnected from the power supply, carefully removed and stained by submersing in GR-Green nucleic acid gel stain solution (Lab Suppy Mall, InnoVita Inc) for 15-30 min. After staining, the DNA bands were visualised under UV illumination using a SYNEGENE VACUTEC UV illuminator fitted with Synoptics 2.0 MP camera made compatible with the GENESys Version 1.1.2.0 computer software.

4.3.6 Southern blotting analysis

The Southern blot assay was performed according to SAMBROOK et al. (1989). Genomic DNA (10 µg) from transformed and non-transformed *D. erectum* plants was digested with *Eco*RI (New England Biolabs, UK) and separated on 1% agarose gel. The 5' Cy5-labelled probes were amplified using the following sequences from Thermo Fisher Scientific (USA):

GUSF: AGGTGGTTGCAACTGGACAA

GUSR: ATGCCATGTTCATCTGCCCA

The probe size was determined as 372 bp and the amplification was carried out using Phusion polymerase (Thermo Fisher Scientific, USA). The blot was hybridized overnight at 43 ˚C by adding 25 ng μL^{-1} of the probe to 0.5 µL of 20 mg mL^{-1} Salmon sperm (Invitrogen™, USA) in sterile water and this mixture was co-incubated with the Nitrocellulose membrane (Amersham Biosciences, UK). Detection was done with Typhoon Trio^{+++} Imager (Amersham Biosciences, UK).

4.4 Results and Discussion

The use of LBA4404 *Agrobacterium* strain in transformation of a close relative of *Dierama erectum*; *Iris germanica*, resulted in high transformation rates **(JEKNIC et al., 1999)**. The same was also reported in transformation of an ornamental monocotyledonous plant; *Agapanthus praecox* **(SUZUKI et al., 2001)**. These reports prompted the use of this supervirulent strain that would render *D. erectum* amenable to *Agrobacterium* transformation. However, removal of residual agrobacteria after co-cultivation was one of the problems that arose during transformation even when cefotaxime was applied to subsequent regeneration medium during selection of putative transformants. Although this happened infrequently in this study, agrobacterium was at times stubborn to the application of cefotaxime with occasional development of bacterial growth during selection. This occurred when cultures were presumed decontaminated of agrobacteria. In such instances, explants were washed in cefotaxime and transferred to the fresh growth medium also containing cefotaxime every 2 weeks until they were free of bacteria. It must be noted however, that this overgrowth was not a major problem as none of the putatively transformed plants were lost due to repeated treatment with cefotaxime.As highlighted in **Chapter 2**, the T-DNA integration into the host plant is influenced by many factors. It is clear that *Agrobacterium*-mediated transformation involves multiple and complex interacting processes. A step by step optimization of various factors affecting transformation efficiency was done in this study. Thus far, this is the first report of genetic transformation of a plant belonging to the genus *Dierama*. The outlined factors were determined by assaying the *GUS* activity in the tissues of putatively transformed plants before and after selection. Most of the studies in the *Agrobacterium*-mediated

transformation of related species such as *Iris ensata* **(BOLTENKOV et al., 2005)**, *Crocus sativus* **(CHEN et al., 2003; AHAMAD et al., 2014)** focus on using the callus as the start material for genetic transformation. However, this study indicated that more transformation (over 60%) could be achieved through the use of ESAMs **(Figure 4.4 and 4.5)** than when callus clusters were used. A low percent transformation efficiency with callus could be due to the fact that cells being accessible for gene transfer were those that are not suitable for regeneration **(KOMARI et al., 1998)**. The ESAMs were the most useful explants for transformation. It was speculated that cells of the ESAMs were more amenable to *Agrobacterium* and perhaps have more potential for regenerating multiple shoots. Although hypocotyl explants have been reported as the best explants for multiple shoot production **(KOETLE et al., 2010)**, they cannot be useful for *Agrobacterium*-mediated transformation of this species as they are resistant to agrobacteria infection.

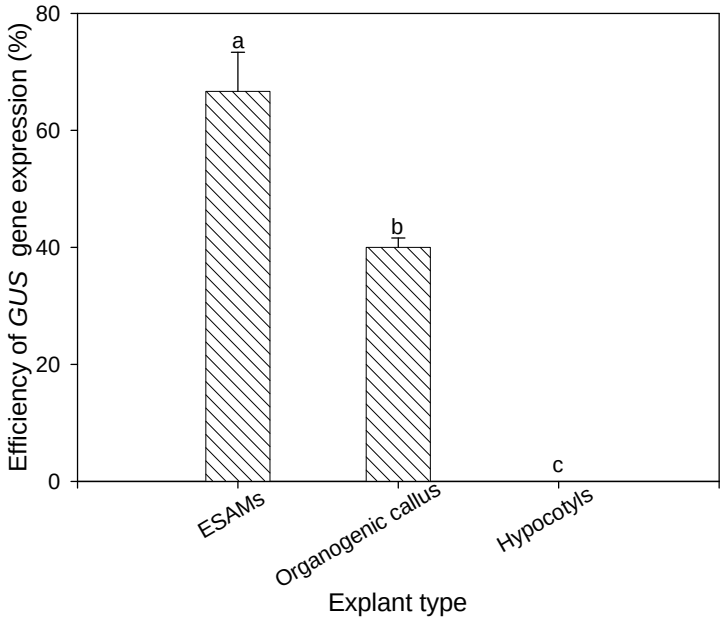

Figure 4.4 Effect of explant type on *GUS* expression in *Dierama erectum*. Bars with different letter(s) indicate significant differences between treatments ($P \leq 0.05$, n = 24) based on Duncan's Multiple Range Test

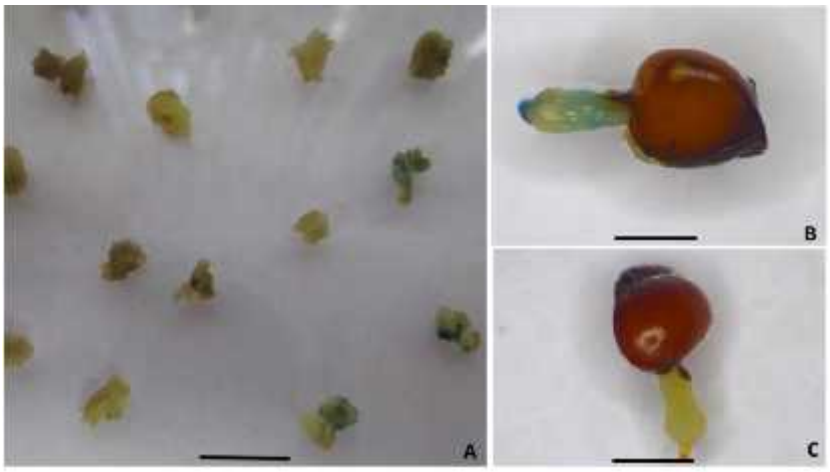

Figure 4.5 Histochemical *GUS* expression on *Dierama erectum* callus clusters (**A**),Bar = 10 mm and transformed ESAM (**B**), Bar = 2 mm; **C** = non-transformed ESAM

The lower transformation efficiencies obtained using the standard methods such as agrobacterial monolayer and suspending explants in *Agrobacterium*, influenced the inclusion of sonication-assisted *Agrobacterium*-mediated transformation (SAAT) as one of the gene delivery systems used in this study. Transformation via SAAT proved to be the most efficient for the ESAM explants as a higher transformation percentage (40 %) could be obtained when SAAT was applied **(Figure 4.6)**. The SAAT treatment was also associated with more multiple shoot formation during regeneration **(Figure 4.7A)**. There was no significant difference between the use of Agrobacterial monolayer system and immersing the explants in Agrobacterial suspension in terms of *GUS* gene expression and shoot regeneration.

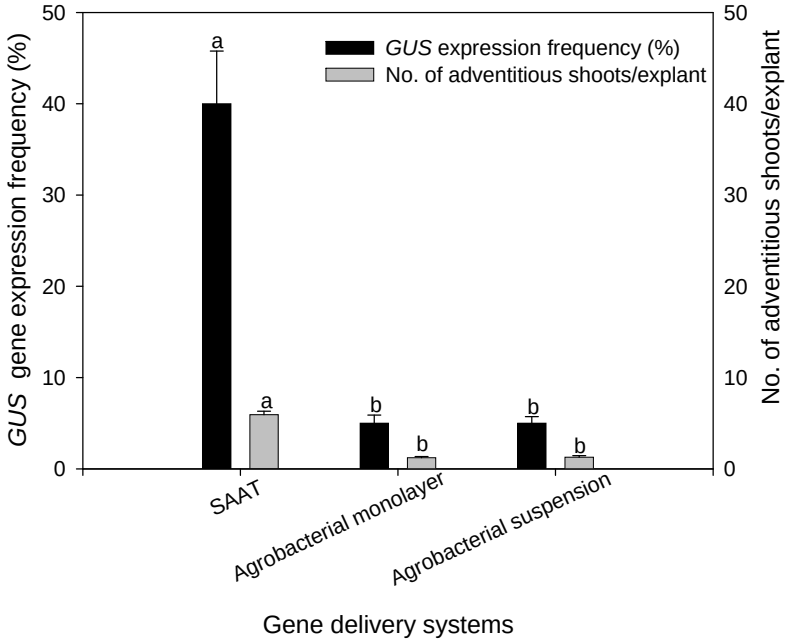

Figure 4.6 Effect of gene delivery systems on *GUS* expression in *Dierama erectum*. Bars with different letter(s) indicate significant differences between treatments ($P \leq 0.05$, n = 24) based on Duncan's Multiple Range Test. SAAT = Sonication-Assisted *Agrobacterium*-mediated Transformation

Figure 4.7 Effect of different gene delivery systems on transformation and regeneration of *Dierama erectum.* Blue color indicates regions of *GUS* activity. **A** = SAAT, **B** = Agrobacterial monolayer, **C** = Agrobacterial suspension. Bar = 5 mm

The use of low frequency of ultrasound (up to 60 kHz) for the enhancement of biotechnological processes has received increased attention. This is a longitudinal pressure wave whose frequency exceeds 20 kHz **(RAICHEL, 2006)**. Recent developments in sonochemistry have made ultrasound irradiation procedures more useful in a broader range of applications **(ROKHINA et al., 2009)**. This low frequency ultrasound acts as an abiotic stress in plants and has many biological effects, hence growth and development of several plants has been stimulated by ultrasound **(TEIXEIRA DA SILVA and DOBRÁNSZKI, 2014)**. The application of ultrasound was first reported by **TRICK and FINER (1997)**, who reported that when target tissues were treated by ultrasound for brief periods in the presence of *Agrobacterium* bearing foreign genes, a 100 to 400-fold increase in transient *GUS* expression could be achieved in a wide variety of species including monocotyledonous plants. By sonicating the ESAMs, chemical and biological processes that enhance the uptake of materials such as PGRs (in this case BA) and hence improved shoot production can be accomplished. This can be attributed to increased cell permeability inflicted by sonication. The micro-wounds or fissures formed after sonication **(Figure 4.8)** increase the chances of *Agrobacterium* to reach and infect target cells. In culture, shoot induction for *D. erectum* is much reliably stimulated when 0.23 mg L^{-1} BA is applied in the growth medium **(KOETLE et al., 2010)**, hence this PGR was included in all the growth media.

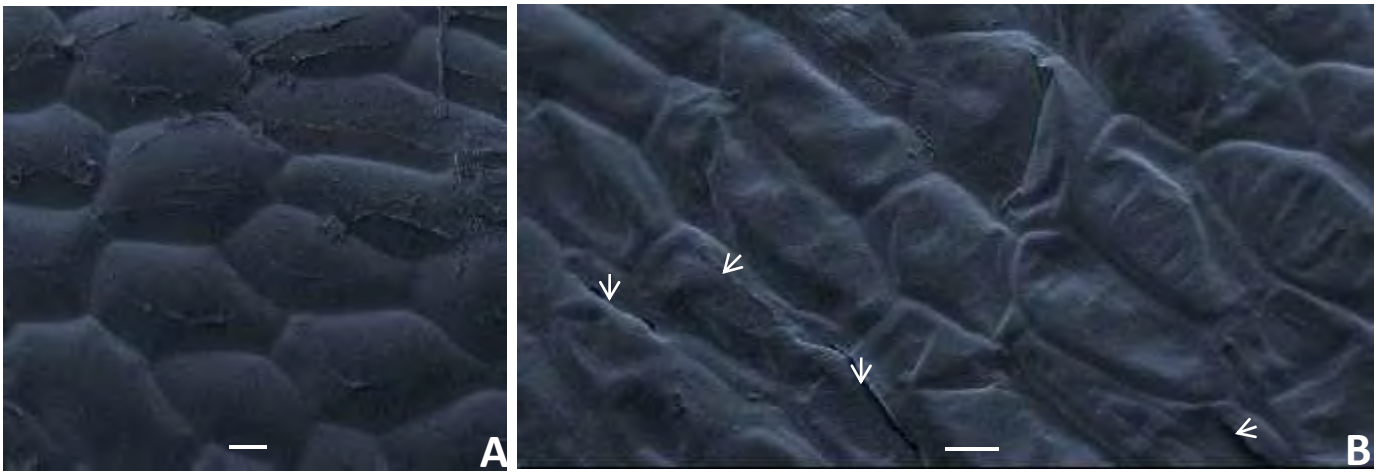

Figure 4.8 The effect of sonication on *Dierama erectum* meristematic tissues. **A** = Non-sonicated tissue, **B** = Sonicated tissue showing fissures (indicated by white arrows), Magnification = 15000 ×, Bar = 10 µm

One of the important factors in transformation systems is the density of the *Agrobacterium* inoculum. The optimized conditions mentioned above (use of ESAMs and SAAT) were used in the subsequent experiments. The plants were inoculated in the *Agrobacterium* suspensions of different optical densities. The results further indicated that transformation efficiency improved with increasing concentration of the *Agrobacterium* and the highest percentage transformation was obtained when an optical density (OD_{600}) of 1.6 was used **(Figure 4.9)**. The higher agrobacterial concentrations beyond OD_{600} of 1.6 resulted in explant death and this was attributed to *Agrobacterium* overgrowth. According to **PARROTT et al. (2002)**, when a pathogen infects the plant, some defence mechanism is triggered; production of reactive oxygen species known as oxygen burst responsible for cell death. Therefore, determining the optimal bacterial inoculation density is important

because with higher OD levels such as 2.0 in the case of *D. erectum*, explant tissues are wholly colonized by the bacteria and its elimination becomes more difficult as the transformation process continues.

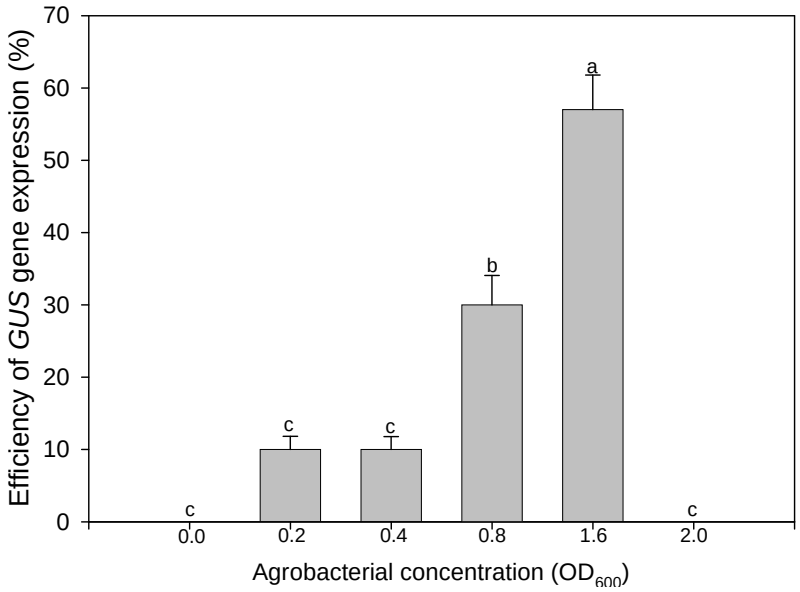

Figure 4.9 Effect of *Agrobacterium* optical density on *GUS* expression in *Dierama erectum*. Bars with different letter(s) indicate significant differences between treatments ($P \leq 0.05$, n = 24) based on Duncan's Multiple Range Test

Plant specific phenolics that induce the expression of the *Vir* gene in *Agrobacterium tumefaciens* are essential for gene transfer **(STACHEL et al., 1985)**. In monocotyledonous plants where these compounds are not naturally synthesized, addition of acetosyringone is usually preferred to enhance gene transfer **(HIEI et al., 1994)**. Using optimal conditions described in the previous experiments (ESAMs, SAAT, OD_{600} = 1.6), the effect of various concentrations of acetosyringone on *GUS* expression were investigated. The results showed that adding acetosyringone to the co-cultivation medium

improved transformation efficiency although not significantly different from the control. Higher concentrations (beyond 50 mg L^{-1}) had a negative effect on the efficiency of *GUS* expression **(Figure 4.10)**. These results are in agreement with other studies where a high transformation efficiency such as that of an ornamental geophyte; *Tricyrtis hirta* was reported when 50 mg L^{-1} was added to the co-cultivation medium (**ADACHI et al., 2005)**. Contrary to the above, a low concentration of 100 µM (19.62 mg L^{-1}) was used to induce virulence in the transformation of *Iris germanica* **(JEKNIC et al., 1999)**.

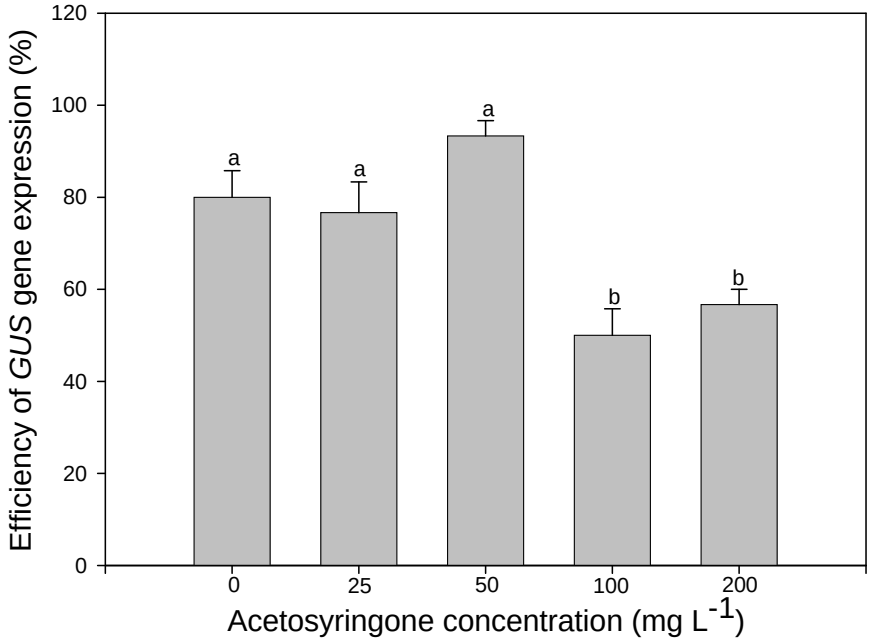

Figure 4.10 Effect of acetosyringone concentration on *GUS* expression in *Dierama erectum*. Bars with different letter(s) indicate significant differences between treatments ($P \leq 0.05$, n = 24) based on Duncan's Multiple Range Test

The co-cultivation duration is another crucial factor influencing gene transfer in *Agrobacterium*-based systems. Again, using the optimized conditions mentioned above

(ESAMs, SAAT, and OD_{600} = 1.6, 50 mg L^{-1} acetosyringone), an experiment investigating the effect of co-cultivation time was conducted. Transformation efficiency was high between 1 and 3 days **(Figure 4.11)**. Leaving the plants in the presence of *Agrobacterium* beyond 3 days led to abundant proliferation of *Agrobacterium* which resulted in tissue necrosis, hindered regeneration and for this reason, the efficiency of *GUS* gene expression was lowered. A co-cultivation period of 2-3 days resulted in enhanced transformation in *Agapanthus praecox* **(SUZUKI et al., 2001)**. The same was reported for *Typha latifolia* **(NANDAKUMAR et al., 2004)**. In some species like *Gladiolus hybridus* however, prolonged co-culture of 12 days led to significantly high transient *GUS* expression rate (50.5 %) when compared to a normal 3-day co-cultivation period without any detrimental effects of agrobacteria **(WU et al., 2015)**.

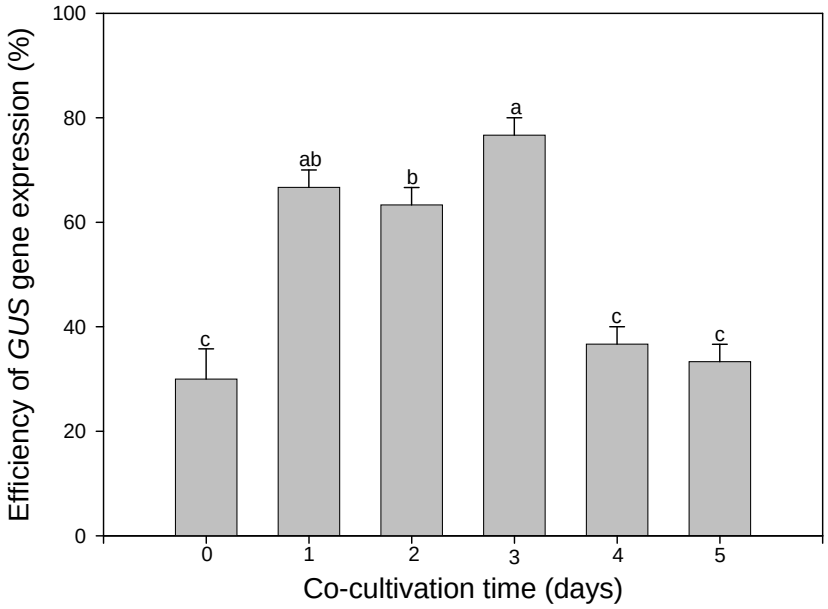

Figure 4.11 Effect of co-cultivation time on *GUS* expression in *Dierama erectum*. Bars with different letter(s) indicate significant differences between treatments ($P \leq 0.05$, n = 24) based on Duncan's Multiple Range Test

Using the optimal transformation procedures, the ESAMs were inoculated in the *Agrobacterium* suspension (OD_{600} of 1.6) and sonicated for 30 s, then left in the suspension for 30 min. They were co-cultivated for 3 days. When the ESAMs were inoculated on selection medium immediately following co-cultivation, these explants appeared to suffer stress inflicted by combined *Agrobacterium*, sonication and kanamycin. To avoid this problem, the explants were transferred to a pre-selection medium (recovery phase) containing no kanamycin (as explained in **Table 4.2**) for 7 days. After the recovery phase, shoots were transferred to a selection medium (as explained in the materials and methods) for 60 days, with 15 days sub-culture intervals. The ESAMs

surviving on the selection medium produced multiple shoots which expressed *GUS* in their leaves. These were rooted and hardened.

Genomic DNA was extracted from transformed and non-transformed plants **(Figure 4.12)**. The PCR amplification of the *GUS* region was performed to detect the presence of the transgene in the T_0 transformants. Out of the 17 plants which histochemically expressed *GUS*, six (6) namely SAAT 1, 2, 3, 5, 10 and 17 were *GUS* positive however, samples 10 and 17 were slightly visible under illumination but very faintly so **(Figure 4.13).**

To confirm stable integration of T-DNA into *Dierama erectum* genome, Southern blotting was applied to analyse DNA isolated from plants identified as positive via PCR as well as non-transformed plants. The positive controls in lanes 2 to 10 (Figure 4.14) displayed positive signals as expected, whereas no positive hybridization signals were obtained with both transformed and non-transformed plants (lanes 11 to 15). These results indicate that the *GUS* gene in *D. erectum* may have been transiently expressed and was not inserted into chromosomes of the transformed plants. This failure of *GUS* to integrate stably into *D. erectum* is inconsistent with the findings by WU et al. (2015), where *Gladiolus hybridus* (a close relative of *Dierama*) was successfully transformed and had *GUS* gene well integrated in its genome. Transformation by *GUS* in *G. hybridus* was driven by the same constitutive promoter (CaMV35S) used in this study. Overall, this could mean that for more successful stable transformation of *D. erectum*, promoters that are more compatible with this monocotyledonous geophyte are a necessity.

Figure 4.12 Acclimatized non-transformed (**A**) and transformed SAAT plants (**B** and **C**)

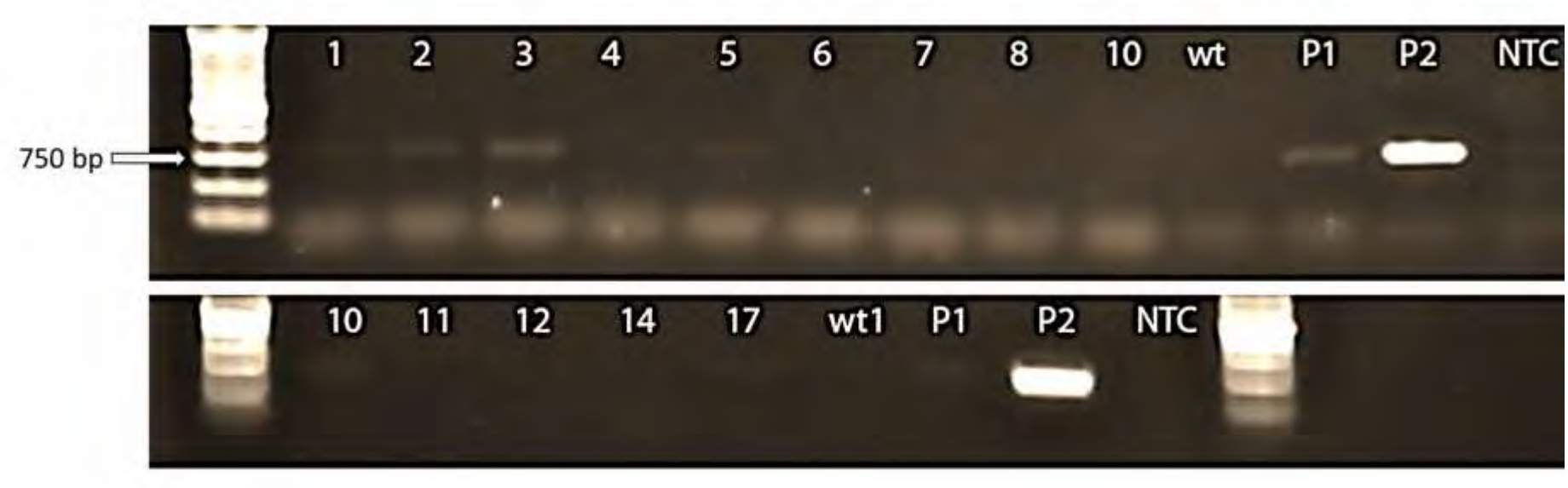

Figure 4.13 *GUS* gene integration. Lanes 1-17 = DNA samples from SAAT-derived plants; P1 and P2 = pCAMBIA 1301 plasmid; NTC = No template DNA controls; Wt and Wt1 = Wild type DNA templates

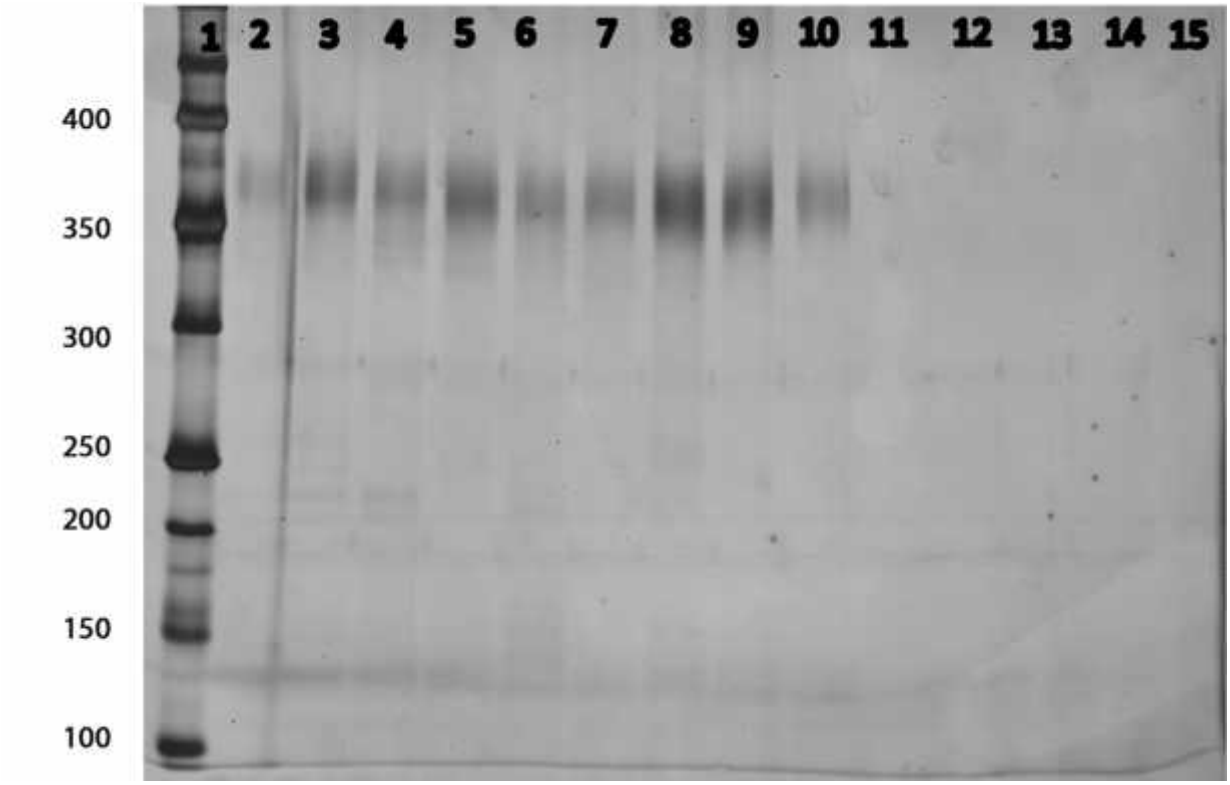

Figure 4.14 Southern blot analysis for *Dierama erectum* transformation. Lane 1 = marker, lanes 2 - 5 = pCAMBIA1301, lanes 6 – 10 = pDLK2:*GUS,* lanes 11 – 14 = transformed plant samples, lane 15 = Non-transformed plant sample

4.5 Summary

In conclusion, this study has accomplished and optimized conditions necessary for effective *Agrobacerium*-mediated transformation in *Dierama erectum*. The study has established that explant types, gene delivery systems, optical density and co-cultivation period influence the probability of T-DNA delivery. The SAAT has proven to be the most efficient system for gene delivery since it gave rise to more plants transiently expressing *GUS* while at the same time maintaining high multiple shoot regeneration. By optimising conditions for gene delivery, transgenic plants resistant to kanamycin were generated. After determining all the factors necessary for efficient transformation of *D. erectum*, the MS media composition are summarised in **Table 4.2**.

It was shown by PCR analysis that the *GUS* gene was present in the T_0 transgenic plants. This is a pioneering report of *Agrobacterium*-mediated transformation of *D. erectum*. Transient GUS gene in the plant material was observed and this proves that *Dierama erectum* is amenable to *Agrobacterium* transformation and this will greatly facilitate the wide-spread transformation of many related species.

Table 4.2 The Murashige and Skoog media and supplements used for genetic transformation of *Dierama erectum*

MS media type	MS media composition
Germination	1/10 strength MS
Co-cultivation	MS + 0.23 mg L^{-1} BA + 50 mg L^{-1} Acetosyringone
Pre-selection	MS + 0.23 mg L^{-1} BA + 50 mg L^{-1} cefotaxime
Selection	MS + 0.23 mg L^{-1} BA + 100 mg L^{-1} cefotaxime + 100 mg L^{-1} kanamycin
Rooting	MS + 0.20 mg L^{-1} IBA + 100 mg L^{-1} cefotaxime + 100 mg L^{-1} kanamycin

CHAPTER 5
TRANSFORMATION OF *DIERAMA ERECTUM* WITH THE EARLY FLOWERING *BpMADS4* GENE

5.1 Introduction

Plant *MADS*-box genes play an important role in a wide range of plant developmental processes; especially the transition from the vegetative to reproductive phase and determination of floral meristem identity **(GOLOVESHKINA et al., 2012)**. The *MADS*-box family has about 107 members most of which have been identified in *Arabidopsis* **(HEIJMANS et al., 2012)**. These genes have been isolated from many plant species and members of each of their clade share similar expression patterns **(ROUKOLAINEN et al., 2010)**. For instance, overexpression of *MdMADS2* and *MdMADS5* (from *Malus* × *domestica*) genes caused early flowering in transgenic *Nicotiana tabacum* **(SUNG et al., 1999)** and *Arabidopsis thaliana* **(KODOTA et al., 2002)** respectively.

The *BpMADS4* isolated from *Betula pendula* is a member of the *APETALA1/FRUITFULL* group of the *MADS* genes, and was found to induce early flowering in apple **(FLACHOWSKY et al., 2007)**. Not only does *BpMADS4* influence flowering patterns, but also the transcription factors regulating senescence and dormancy processes in *Populus tremula* (**HOENICKA et al., 2008)**. The expression is known to start at an early stage of male and female inflorescence development. The earliest line of *B. pendula* flowered 11 days after rooting and the plants transformed with the *BpMADS4* antisense construct did not show any sign of flower development in 2 years **(ELO et al., 2007)**. These studies show that *BpMADS4* plays a critical role

in the transition from vegetative to reproductive development and provides a promising tool for accelerating flowering in various plant species **(ELO et al., 2001)**. Further investigations of *MADS*-box transcription factors can be used to explain the genetic regulation of flower morphology diversity among different species and even within a single genotype **(GOLOVESHKINA et al., 2012)**. This study was aimed at investigating the possibility of integrating the *BpMADS4* gene into the *Dierama erectum* genome with the intention of obtaining early flowering genotypes.

5.2 Materials and Methods

5.2.1 *Agrobacterium tumefaciens* preparation

The *Agrobacterium tumefaciens* strain (LBA4404) containing *453p9N-35S-BpMADS4* vector construct shown below **(Figure 5.1)**, was a kind donation from Dr Henryk Flachowsky (Dresden, Germany). It was sent as a stab culture and upon arrival, was plated using a three way dilution streaking on Luria Broth (LB) medium containing kanamycin, rifampicin, and spectinomycin all at the concentration of 0.1 mg mL^{-1}. The plates were incubated at 28 ˚C until the appearance of single colonies on agar solidified bacterial medium. Afterwards, single colonies from the plates were inoculated and grown until the log phase in liquid LB medium containing the aforementioned antibiotics until the log phase was reached. The *Agrobacterium* was cryopreserved by using 500 µL bacterial suspension mixed with 500 µL glycerol. The *Agrobacterium* cryovials were placed in a -70 ˚C freezer until further use.

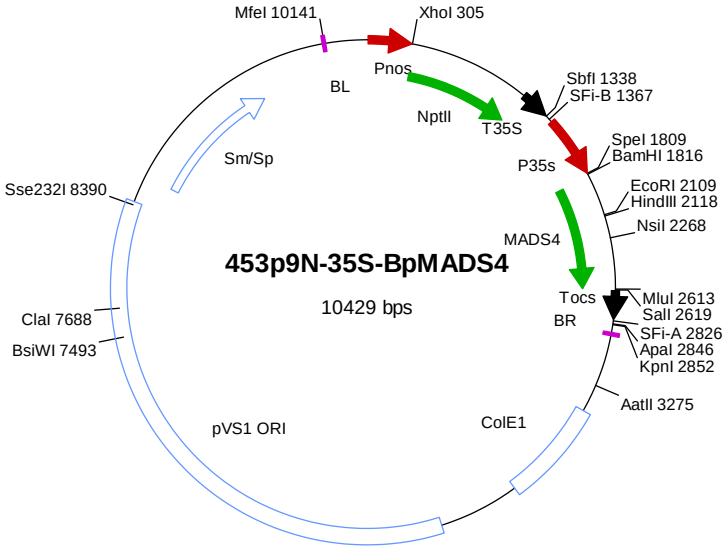

Figure 5.1 The *453p9N-35S-BpMADS4* vector map showing the kanamycin (*NptII*) and spectinomycin (Sm/Sp) resistance sites, *35S* promoter and the *MADS4* regions **(FLACHOWSKY et al., 2007)**

5.2.2 *Agrobacterium*-mediated transformation

Unless otherwise stated, the conditions optimised in **Chapter 4** were used in the subsequent experiments in the current study. A fresh liquid culture of *Agrobacterium* was utilised for the transformation. An overnight culture grown on LB medium with appropriate antibiotics was transferred into centrifuge tubes under laminar flow conditions and centrifuged at 5000 × *g* in an Avanti™ J-25-I centrifuge (Beckman, USA) using a JA-14 rotor for 10 min. The pellet was re-suspended in the LB medium without antibiotics after the supernatant was discarded. The centrifugation was repeated to remove antibiotics and the pellet was diluted in fresh LB medium free of antibiotics. Embryonic shoot apical meristems (ESAMs) were excised and sonicated for 30 s in the *Agrobacterium* suspension (OD_{600} = 1.6). Explants were blotted dry on sterile filter paper to prevent excessive agrobacterial growth, and inoculated on co-

cultivation (MS + 0.23 mg L^{-1} BA + 50 mg L^{-1} acetosyringone) medium for 3 days. Bacterial growth at the edges of the explants was evident after this period. Explants were washed in cefotaxime (350 mg L^{-1}) for 20 min with occasional agitation and rinsed 3 times in sterile distilled water. After blotting dry on sterile filter paper, they were transferred to a pre-selection phase medium (MS + 0.23 mg L^{-1} BA + 50 mg L^{-1} cefotaxime) for 7 days to allow explant recovery from sonication and bacterial infection. They were washed again in cefotaxime, rinsed and blotted dry. Only explants with no signs of necrosis or browning tissue were transferred to selection medium (MS + 0.23 mg L^{-1} BA + 100 mg L^{-1} cefotaxime + 100 mg L^{-1} kanamycin). Twenty Petri dishes containing 10 explants each were used for selection. Explants were left to regenerate and transfer to fresh medium was done every two weeks until the cultures were 12 weeks old. Multiple shoots obtained from these cultures were separated and grown on rooting medium (MS + 0.20 mg L^{-1} IBA + 100 mg L^{-1} cefotaxime + 100 mg L^{-1} kanamycin). Roots were visible after 5 weeks. After 12 weeks, plants were acclimatized as detailed in Section 4.2.5.

5.2.3 Effect of photoperiod cycles and phenotypic analysis of putative transformants

Putative transformants were selected from 1-month-old acclimatized stock plants. They were left to grow under different photoperiod regimes (16h light/ 8 h dark for 6 months served as control, 16 h light/ 8 h dark (long day (LD)) and 8 h light/ 16 h dark were done interchangeably in 3 monthly cycles) to trigger flowering over 6 months. That is, plants grown in LD were transferred to SD cycles after 3 months and vice versa. Each treatment consisted of 10 individually potted plants. Plant growth was maintained in temperature-controlled Conviron® growth cabinets (Controlled

Environments Ltd, Winnipeg, Canada) set to 25 ˚C with 16 h or 8 h photoperiods and an irradiance of 151 µmol m^{-2} s^{-1} (provided by cool fluorescent tubes (110 W, Phillips, USA; 75% total wattage). Data was recorded after every 3 months and analysed.

5.2.4 Detection of *BpMADS4* gene by PCR

Genomic DNA was extracted from healthy acclimatized plant leaf tissues and the plasmid was isolated using the procedures outlined in **Appendix 4.2**. Transformation of putative lines was confirmed using PCR-mediated amplification of *BpMADS4* primers. The primer set used in this study was synthesised by Inqaba Biotec (Pretoria, South Africa). These primers were delivered as lyophilised powders and were suspended in ultra-pure nuclease-free water upon arrival and prepared as 100 µM stock solutions and then stored at -20 ˚C until required.

Before the amplification process, the integrity of all the plasmid DNA was analysed using agarose gel electrophoresis in 2% agarose gel (w/v). The loading dye (5 µL) was used to track the electrophoresis progress. The gel was run at 7 volts/cm at room temperature and the DNA was visualised by GR-Green staining (Lab Suppy Mall, InnoVita Inc). The band size was determined by comparing them to the Thermoscientific GeneRuler DNA ladder (#SM0243). The DNA bands were visualised under UV illumination using a SYNEGENE VACUTEC UV illuminator fitted with a Synoptics 2.0 MP camera made compatible with the GENESys Version 1.1.2.0 computer software. After this, only the plasmid DNA sample of highest integrity was used in the following PCR procedure.

The PCR mixture contained 1.0 µg of plant genomic DNA, 25 µL PCR 2X Taq Master Mix (Bioteke Corporation), 1.0 µM forward primer, 1.0 µM reverse primer, and nuclease free water to a total reaction volume of 50 µL. For plasmid DNA, the concentration was 0.05 µg. The PCR reaction was performed by denaturation at 94 ˚C for 5 min, followed by 30 cycles of 1 min denaturation at 94 ˚C, 30 s annealing at 56 ˚C and 1 min extension at 72 ˚C. After a final extension at 72 ˚C for 5 min, the amplified DNA fragments were separated on 2% agarose gel. The expected fragment length of the *BpMADS4* after amplification was 782 bp. The primers (FLACHOWSKY et al., 2007) used were as follows:

*BpMADS4*_F 5′-TAG GGT TCA GCT TAA GCG AAT-3 (T*m*; 58.66)′

*BpMADS4*_R 5′-GCC GGA TCA CGG TTA TCC GAG-3 (T*m*; 66.47)′

For electrophoresis, 20 µL of amplified sample was dispensed into a gel slot. In a separate well 10 µg DNA ladder mix was added. Samples were electrophoresed at 7 volts/cm (56 V in total), until the dyes migrated to the end of the gel nearest to the positive electrode.

5.2.5 Particle bombardment transformation of *Dierama erectum* with *BpMADS4* gene

The plasmid DNA was acquired using the *Agrobacterium* plasmid isolation procedure outlined in **Section 4.3.2**. An experiment was designed to assess the effect of plasmid DNA concentration on transformation of *D. erectum* using ESAMs as explants.

5.2.6 Preparation of microparticles

Fifty (50) mg of 1.1 µm tungsten particles were incubated in 70% (v/v) ethanol overnight. Particles were then washed by centrifuging at 10 000 × *g* in a desk-top centrifuge. The supernatant was removed prior to re-suspension of particles in sterile distilled water by vortexing. They were washed twice and re-suspended in 1 mL of 50% (v/v) sterile glycerol solution. For plasmid DNA precipitation onto the particles, 25 µL of the tungsten suspension was added to different microfuge tubes and DNA stock was pipetted such that the DNA concentrations were 5, 10 and 15 µg μL^{-1}. Twenty five (25) µL of 1 M $CaCl_2$ and 10 µL of sterile deionised water were added sequentially. The tube contents were mixed thoroughly by gently vortexing and then left on ice for 10 min to allow for precipitation and sedimentation. After 10 min, the supernatant was removed and the DNA particles at the bottom of the tube were then re-suspended by vortexing of the tube. Five (5) µL of the suspension was used per shot of the target tissue.

5.2.7 Preparation of the target tissue

Between 10 to 16 h prior to gene delivery, the ESAMs were arranged on regeneration medium (MS + 3% (w/v) sucrose + 0.23 mg L^{-1} BA) in a circle at the centre of the Petri dish. There were 20 Petri dishes with 12 explants per plate for each bombardment. This experiment was repeated twice.

5.2.8 Conditions for biolistic gene transfer

All the GENEBOOSTER^{TM} (ELAK Ltd. Co., Hungary) accessories were cleaned thoroughly by spraying with 70% ethanol (v/v) 1 h before bombardment. The stopping plate mesh was autoclaved prior to use and it was then fitted onto the stopping plate before firing. The macroprojectiles were stored in a jar filled with absolute ethanol at least 16 h before use. These were placed on a sterile Petri dish and left to evaporate under the laminar flow. Once the macroprojectiles had dried, the DNA/tungsten mixture (5 µL) was pipetted onto the centre of the top of the macroprojectile. The macroprojectile was inserted into the acceleration barrel. For each shot, the Petri dish containing target tissue was opened and placed on the 4th shelf (from the top) of the vacuum chamber and locked inside. The tissue was bombarded by DNA-coated microparticle. Throughout the entire experiment, bombardment conditions were kept constant. The distance of the stopping plate from the target tissue was 8 cm, the vacuum pressure was -40 kPa and the tissue was bombarded with the gas pressure of 4000 kPa.

After the shots, the Petri dishes were sealed and transfer to a 16 h light growth room with a temperature range of 25-26 ˚C. After 13 days (when the tissue started to show signs of regeneration), explants were transferred to a selection medium (MS + 0.23 mg L^{-1} BA + 100 mg L^{-1} kanamycin) for selection of putative transformants.

5.3 Results and Discussion

5.3.1 Effect of photoperiod and phenotypic analysis of putative transformants

Table 5.1 shows the effect of different photoperiods on growth rate and plant length of *D. erectum* putative tranformants observed over a period of 6 months. Plants were rotated between 8 h light (SD) for the first 3 months and 16 h light (LD) for the last 3 months or vice versa.

In the first 3 months, plants grown under SD grew slower (about 3 mm per week) than the plants maintained under LD; this being attributed to limited light required for photosynthesis and optimal growth. Growth rate increased slightly when plants were transferred to LD conditions following 3 months of SD treatment **(Table 5.1)**. In all the treatments, plants attained the second leaf stage at 3 months Plant height reached about 15 cm after 6 months for both LD and SD acquired plants and this was significantly lower than the control (22.90 ± 0.460 cm). By the end of the 6 month observation period, the third leaf had appeared in all treatments. However, no flower competent stage was observed over this period.

Flowering of 3 *Watsonia* species: *W. borbonica*, *W. pillansis* and *W. tubularis* was observed when these plants were grown under SD conditions especially after the development of the third leaf. The microscopic examination of the shoot apical meristem revealed that appearance and extension of the second leaf signified the anatomical transition to flowering **(THOMPSON et al., 2011)**. This was not the case in the current study even after the extension of the third leaf. There is a major difference between the leaf morphology of *Dierama* and *Watsonia*. The *Watsonia* leaves are

much broader while those of *Dierama erectum* are thinner (grass-like). It is therefore possible that in *Dierama*, to attain a reproductive phase, a much bigger plant size than the one observed **(Figure 5.2)** may be a prerequisite.

In plants, transition from vegetative growth to flowering involves a major change in the shoot apex development. This can occur once in annual species, or repeatedly in perennials such as *Dierama erectum*. Perennials flower in consecutive years while maintaining vegetative development after flowering, whereas in annuals, flowering is usually associated with senescence and death of the whole plant.

In contrast to annuals, the molecular mechanisms controlling flowering in perennials has not been extensively studied and is therefore poorly understood. The major factor preventing detailed studies in perennials is that often, only a part of the plant responds to flower inductive signals. Thus, only a subset of meristems become reproductive. Therefore with perennials, reproductive competence varies between meristems such that when they are exposed to conditions that favour flowering, only competent meristems perceive flower inductive signals and hence differentiate into inflorescences and flowers **(BATTEY and TOOKE, 2002)**.

Table 5.1 Effect of different photoperiod regimes on growth of *Dierama erectum* resulting from *Agrobacterium*-mediated transformation with *BpMADS4*.

Photoperiod regime	Average growth rate (mm/week)		Plant height above soil level at 6 months (cm)
	< 3 months	3-6 months	
Continuous LD for 6 months (control)	5.70 ± 0.540[a]	6.40 ± 0.400[a]	22.90 ± 0.460[a]
LD for the first 3 months	5.70 ± 0.520[a]	---	---
SD for the first 3 months	3.30 ± 0.300[b]	---	---
LD for the last 3 months	---	4.50 ± 0.400[b]	15.00 ± 0.39[b]
SD for the last 3 months	---	3.00 ± 0.210[b]	15.10 ± 0.53[b]

Values in columns with different letter indicate significant differences between treatments ($P \leq 0.05$, $n = 10$) based on Duncan's Multiple Range Test

Figure 5.2 Putatively transformed *Dierama erectum* plants after 6 months acclimatization

5.3.2 Detection of *BpMADS4* gene by PCR

It must be highlighted that after electrophoresis, it was difficult to obtain clear bands with 1% (w/v) agarose. The agarose concentration was then increased to 2% and this gave better results. Again, the choice of PCR Master Mix was an important factor. The PCR Master Mix obtained from Bioteke Corporation (2X Taq Master Mix) gave better results than the Fermentas Master Mix.

Although an efficient transformation system recorded in the previous **Chapter 4** was envisioned to have formed a basis for integration of other genes such as the *BpMADS4*, the efficiency of gene incorporation based on PCR evidence was very low **(Figure 5.3)**. Comparing the bands between the wild type and all other *BpMADS4* putative transformants, it was concluded that the T-DNA had integrated into line 3

(Figure 5.3). However, the *BpMADS4* could not be expressed at a functional level; that is, no early flowering on the species was recorded in the period of the 18 months duration of the study. This transient gene expression could be due to various reasons such as subsequent regeneration or survival of non-transgenic tissues during selection **(BHAT and SRINIVASAN, 2002)** which may have led to the occurrence of escapes or false positives. There is also a possibility of the *NptII* gene not being transferred concurrently with the *BpMADS4*, thus resulting in plant survival on kanamycin-rich medium without the expression of the *BpMADS4* gene.

As highlighted in the literature **(Chapter 2)**, relatively little is known regarding the mechanisms that incisively control mechanisms of T-DNAs into the host genome of Iridaceae species. In fact, most assays performed for transient gene expression fail to correlate with expression of stably incorporated transgenes. The level of transient gene expression often does not match expression of stably integrated genes **(GELVIN, 2000)**, indicating that T-DNA successfully transferred to the *D. erectum* genome in this case did not stably integrate and subsequently failed to express.

Nevertheless, recent developments promise more rapid progress **(KRENS and KAMO 2013)**. There has been a focus on a small number of species that are generally susceptible to genetic transformation with MADS-box genes. For instance, overexpression of flowering genes such as *APETALA1, LEAFY* and *FRUITFUL* (*CiFT*) that regulate flowering in *Arabidopsis thaliana*, have shown to overcome the juvenile phase in transgenic plants of citrus within 3-22 months **(PENA et al., 2001; ENDO et al., 2005)**. This was however not the case with *D. erectum* since plants did not reach the reproductive phase for the duration of the study that lasted 29 months (this includes

an additional 11 months where plants were left to grow in the green-house). An attempt to alternate the photoperiodic regimes also failed to trigger *BpMADS4* expression indicating that positive gene integration shown by PCR **(Figure 5.3)** could have only been transient and therefore the gene could not be expressed at functional level as was expected.

Figure 5.3 *BpMADS4* integration into *Dierama erectum*. Lanes 1= wild type; 2-7 = DNA samples from putatively transformed plants; P = 453p9N-35S-*BpMADS4* plasmid; N = No template DNA (control); M = DNA ladder

5.3.3 Particle bombardment transformation of *Dierama erectum* with Bp*MADS4* gene

The second part of this study was an attempt to integrate *BpMADS4* gene into *D. erectum* genome by particle bombardment. This was not successful since all bombarded embryogenic shoot apical meristems did not survive during the selection phase **(Figure 5.4)**. This was an indication of complete failure of gene integration and hence transcription. It is possible that a number of factors determined this outcome and these include:

- Instances where helium gas used would decrease in pressure rendering gas pressure decrease in the vacuum shaft and thus lowering the speed of the macro-particles required to penetrate the plant tissue;
- The use of tungsten particles as opposed to gold could have been a disadvantage since tungsten can catalytically degrade DNA **(KIKKERT, 1993)**; and
- Breakage of insert DNA fragment imposed by particle bombardment. This has been reported especially with long DNA fragments **(HANSEN and WRIGHT, 1999)**.

Some alterations in the standard biolistics protocol including pre-culture of the plant tissue, and subjecting it to the medium containing an osmotic regulator such as sucrose (see **Section 5.2.5.2**) to improve gene delivery **(HANSEN and WRIGHT, 1999; VAIN et al., 1993)**, did not yield any improvement in transformation.

Figure 5.4 Tissue necrosis on selection medium after particle bombardment with *BpMADS4* plasmid in *Dierama erectum*. **A** = Preliminary signs of necrosis at the base of explant tissues after 3 weeks. **B** = Complete explant death after 5 weeks. Bar = 1 cm

5.4 Summary

Positive early flowering expression reported through integration of *BpMADS4* gene in various plant species prompted the interest of introducing it to *Dierama erectum*; a monocotyledonous ornamental geophyte. Despite the disappointing results of failure to express *BpMADS4* at a functional level, this part of the study showed the possibility of introducing genes of interest into *D. erectum* and these results warrant further investigations. An attempt to introduce this gene through particle bombardment was also unsuccessful. It is speculated that this was due to an array of factors and these must be considered important in any plant genetic transformation programme.

CHAPTER 6

GENERAL CONCLUSIONS

Prior to the commencement of this study most reports available for genetic transformation in Iridaceae was for *Gladiolus* species **(KAMO et al., 2010)**. Added to the list is now a pioneering report of *Agrobacterium*-mediated transformation in the genus *Dierama*. Firstly, the *in vitro* establishment of organogenic callus showed some potential of acting as starting material for *Agrobacterium*-mediated transformation. However, the efficiency of transformation could be improved if ESAMs are used in place of callus.

Over and above the listed factors in **Chapter 2** known to affect genetic transformation in monocotyledonous geophytes, it was found that sonication (mechanical treatment) could positively affect *Agrobacterium*-mediated transformation as shown in **Chapter 4**. An efficient transformation system is the one which offers simple steps for generation of plants expressing a gene of interest while at the same time maintaining shoot proliferation and this was well met through the use of SAAT.

Molecular analysis through PCR amplification, selection of putative explants on kanamycin-rich medium as well as *GUS* assaying, convincingly showed that *D. erectum* can be modified genetically. The results obtained draw assumption that mechanisms of T-DNA transfer regulated by *Agrobacterium* 'machinery' functions well despite *D. erectum* being a monocotyledonous geophyte.

***In Vitro* Methods**

Germinate decontaminated seeds on 1/10th strength MS solidified with agar

Isolate embryonic shoot apical meristems immediately when seeds start germinating

Transform ESAMs using SAAT (30 s sonication in Agrobacterial suspension (OD600 of 1.6, co-cultivate for 3 days and refer to **Table 4.2** for pre-selection, selection and rooting)

Acclimatize putative transformants

Confirm transformation by PCR and Southern blot hybridization

Figure 6.1 A schematic illustration for *Agrobacterium*-mediated transformation of *Dierama erectum*

The discovery of new genes has raised interest in their study in different species **(XU et al., 2012)** and this includes early flowering genes. The *MADS*-box genes such as *BpMADS4* have been found to promote early flowering in various plants and this encouraged the use of *BpMADS4* in this study. Although the PCR results revealed its low transient expression in *D. erectum*, future studies of this nature would provide a further insight into the regulation of *BpMADS4* gene in transgenic cultures. Perhaps the use of promoters that are more compatible with this plant can lead to more success

in transforming this plant. Instead of using the CaMV 35S like in this study, promoters such as Mannopin synthase, RolD, Ubiquitin or LAT52 (DUTT et al, 2014) can be utilised to take control and thereby increasing gene expression. Another route would be to further investigate the conditions that favour embryogenic callus formation to assist genetic transformation and hence more gene integration as was recently done in *Gladiolus hybridus* by WU et al. (2015).

As a final note, this thesis aimed at developing callus induction protocol to assist genetic transformation of *D. erectum*. This was partially met by obtaining organogenic callus and genetic transformation via this route was proved possible. The second major objective of the study has been achieved as transformation of *D. erectum* was attained, thus showing that this monocotyledonous ornamental geophyte is amenable to *Agrobacterium*-mediated transformation; a first time successful attempt in the genus *Dierama*.

APPENDICES

Appendix 4.1

Embryonic shoot apical meristem (ESAM) preparation for viewing under conventional scanning electron microscope (SEM)

4.1.1 Fixation

Primary Fixation: ESAMs were immersed in 3 % buffered glutaraldehyde for 1 h

Buffer Wash: They were washed 3 times for 5 min in 0.05 M sodium cacodylate buffer

Dehydration: Explants were dehydrated in 10, 30, 50, 70 and 90% ethanol series, each for 10 min and then 3 times for 10 min in 100% ethanol

4.1.2 Critical Point Drying (CPD)

Explant samples were transferred to CPD baskets under 100% ethanol. During the drying process, the ethanol was replaced with liquid carbon dioxide (CO_2). The CO_2 was heated and pressurised to its critical point at which it was converted to gas (at this critical point, both liquid and gas have equal densities) without the damaging effects of surface tension on the samples. This procedure was done using a Critical Point Drier (Quorum K850).

4.1.3 Mounting

The dried samples were mount carefully on the SEM stubs.

4.1.4 Coating

Stubs with samples were transferred to the ion coater (EIKO 1B·3). At this stage, samples were made conductive to the electron beam by coating with gold particles.

4.1.5 Viewing

Dried coated samples were viewed with a SEM (ZEISS EVO/LS15) fitted to a secondary electron detector compatible with SmartSEM V05.04.02.00 computer software.

Appendix 4.2

4.2.1 Plant DNA extraction

CTAB-extraction solution: 2% (w/v) CTAB; 100 mM Tris-HCl pH 8.0; 20 mM EDTA pH 8.0; 1.4 M NaCl

CTAB/NaCl solution: 10% CTAB; 0.7 M NaCl mixed while heating at 65 °C and stirring

CTAB-precipitation solution: 1% w/v CTAB; 50 mM Tris-HCl pH 8.0; 10 mM EDTA

High-salt TE buffer: 10 mM Tris-HCl pH 8.0, 0.1 mM EDTA pH 8.0; 1 M NaCl

TE buffer: 10 mM Tris-HCl pH 8.0; 1 mM EDTA pH 8.0

Chloroform: iso-amyl alcohol (24:1 ratio)

80 and 100% (v/v) ethanol

100% Isopropanol

7.5 % (v/v) 2-Mercaptoethanol

3% (w/v) Polyvinylpoly-pyrolindone

4.2.2 *Agrobacterium* plasmid isolation

Luria broth medium supplemented with appropriate antibiotics

Cell suspension solution: 50 mM glucose, 25 mM Tris-HCl, 10 mM EDTA (pH 8.0)

20 mg/ml lysozyme solution

Cell lysis solution: 0.2 M NaOH, 1.0 % SDS

Phenol solution

Neutralization solution: 3 M Na-acetate (pH 5.2)

TE buffer: 10 mM Tris-HCl, 0.1 mM EDTA (pH 7.8)

4.2.3 TAE solution for gel electrophoresis (50X)

121 g Tris

28.55 mL Glacial acetic acid

50 mL of 0.5 M EDTA

421 mL Distilled water

2 g Agarose

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