# *In vitro* Polyploidization of Selected indigenous Plant Species

by

**Viloshanie Reddy**

Submitted in fulfilment of the academic requirements for the degree of

Master of Science

School of Life & Environmental Sciences

University of KwaZulu-Natal, Durban, South Africa

January 2005

## Preface

The experimental work described in this dissertation was carried out in the School of Life and Environmental Science, University of KwaZulu-Natal, Durban, South Africa from January 2001 to December 2005, under the supervision of Professor Michael T. Smith (School of Life and Environmental Sciences).

These studies represent original work by the author and have not otherwise been submitted in any form for any degree or diploma to any tertiary institute. Where use has been made of the work of others it is duly acknowledged in the text.

VILOSHANIE REDDY (27/03/2006)

## Dedication

I dedicate this dissertation to my family for their love and understanding and for everything that they have done for me.

## Acknowledgements

To my supervisor Michael Smith for his guidance throughout my research, to Karin Hannweg and James Vos of the Agricultural Research Council – Institute for Tropical and Subtropical Crop in Nelspruit for their financial assistance and encouragement and being an inspiration to me. I would also like to extend my sincere gratitude to Gerhard for his assistance in flow cytometry analysis.

I am also indebted to other colleagues who have helped me at various stages during the course of this work. To Dr. Vesselina Merhar for her assistance in Transmission Electron Microscopy analysis. To Mrs. Priscilla Maartens for training and assistance in electron microscopy technology. To Denise Berjak for her assistance in darkroom work, I am truly grateful. To all members of the Biochemistry Research Unit for their help during the course of my research.

Lastly I thank God for his divine intervention during this stressful period in my life and for providing me the opportunities that I have been given and for all the kind people that have assisted me during the course of this research work.

## Abstract

Many plant species indigenous to South Africa have ornamental, medicinal and horticultural value. Polyploidization is one technique that has been used to artificially produce superior genotypes, particularly in horticultural species.

In the current investigation two antimitotic substances, colchicine at concentrations of 0.1% and 0.01% and oryzalin at concentrations of 0.01% and 0.001%, were used in an attempt to polyploidize microshoots of *Dorotheanthus bellidiformis* (Burm.f.) N.E.Br and *Mondia whiteii* (Hook.f.) *in vitro*. Microshoots of *D. bellidiformis* and *M. whiteii* obtained from nodal cuttings of *in vitro* germinated seedlings were maintained for 48 hours in liquid medium containing the antimitotic substances and thereafter cultured on sucrose-supplemented MS medium. The treated microshoots were evaluated for elongation, necrosis, contamination and phenolic exudation. Best results were observed in *M. whiteii* microshoots treated with antimitotic substances and transferred on to solid sucrose-supplemented MS medium containing 0.2% activated charcoal for 4 weeks. Leaves from the surviving treated plants were excised and used for flow cytometric analyses to evaluate changes in chromosome number. Shoots of *M. whiteii* treated with 0.01% colchicine showed no changes in chromosome number, while the higher concentration used produced polyploids and mixaploids. However, oryzalin at 0.01% concentrations produced a comparatively higher number of microshoots that were polyploids and mixaploids. Shoots of *M. whiteii* that have altered chromosome number have been transferred onto multiplication medium, for future evaluation of changes in phenotypic characteristics.

The germination response of seeds of *D. bellidiformis* was evaluated in the presence of oryzalin (0.01% and 0.001%) and colchicine (0.1% and 0.01%). Poor germination was observed in seeds germinated in the presence of 0.01% oryzalin. Upon transfer of the germinated seedlings treated with antimitotic substances onto sucrose-supplemented MS medium, subsequent growth and

development was restricted. Shoot and root development was different for the seedlings germinated in the presence of the two antimitotic substances. Shoot elongation and root development was vigorous in seedlings geminated in the presence of 0.01 colchicine and stunted development was observed in seedlings germinated in 0.1% colchicine. On the other hand restricted rootind was observed in seedlings germinated in the presence of oryzalin, and the shoots lacked pigmentation. Meristematic cells excised from the shoot tips of the treated seedlings showed several ultrastructural changes including abnormal mitochondrial development, endomembrane formation and vacuolation.

It was concluded that oryzalin and colchicine influence ultrastructure in plant cells differently. Practical constraints associated with plant tissue culture also influence the rate of *in vitro* polyploidization. Since different plant species require different conditions for optimal growth, it was also noted that no unique polyploidization treatment can be used for a wide range of plant species, individual species require different growth conditions.

# Table of Contents

# CHAPTER ONE
# INTRODUCTION AND LITERATURE REVIEW

## 1.1 INTRODUCTION

### 1.1.1 Indigenous plants

South Africa has an abundant plant species diversity and relatively few of these species are utilized commercially (Coetzee *et al.*, 1999), however there is a need to increase levels of production and marketing of indigenous crop (Okole, 2004). Due to the diverse range of indigenous plants in South Africa, a great potential exists for commercialization (Hannweg, 2004; Okole, 2004). Commercialization of indigenous plants will create employment opportunities for the rural and peri-urban unemployed people of Southern Africa, but at the same time protect over-exploitation from wild populations, maintaining a gene pool, and offer marketing opportunities (Allemann *et al.*, 2004). Currently most of these indigenous plants are utilized for their medicinal, pharmaceutical and ornamental properties, as feed, for traditional medicines, cosmetics, flavourants and ornamental purposes (Okole, 2004). There are over 21 000 known South African plants of which 5000 – 10 000 species have horticultural potential. Many South African plants have been introduced to the horticultural market world-wide, however, South Africa has still much more to offer (van Jaarsveld, 1996). There are many plants that have been overexploited (Fennell and van Staden, 2004; Nigro *et al.*, 2004), but in terms of horticultural and medicinal potential, only a few plant species have been exploited (van Staden, 1998). An introduction of several other indigenous species to the international market will be economically beneficial for South Africa.

### 1.1.2 Utilization of indigenous plants

A few species are used as edible food material. The leaves and roots of edible plants have a high nutritional value and can play an important role in the prevention of malnutrition in rural areas (Allemann *et al.*, 2004; van Rensburg *et al.*, 2004). Some cultivated species such as melons and cowpeas are considered important sources of leafy vegetable, there are several other leafy plant species that have the potential for cultivation (van Rensburg, 2004) and a few species that are considered important edible food material have been commercialized. Indigenous beverage types such as rooibos (*Aspalathus linearis*) and honeybush tea (*Cyclopia* spp.) have developed as an agricultural industry with export potential (Coetzee *et al.*, 1999).

A great diversity of floral wealth exists within the Cape (Fennell and van Staden, 2004) particularly with regard to bulbs, rhizomes and corms and from this floral wealth that exists within the Cape, European explorers have in the past, collected plant material from which a range of horticultural products were developed over the centuries. Plant species such as *Gladiolus* and *Freesia*, originally from South Africa, were successfully introduced into the international cut flower industry (Coetzee *et al.*, 1999). Previously the cut flower industry in the Western Cape was primarily supported by indigenous flowers harvested from its natural habitat. Currently the industry relies on the cultivation of many species. However if cultivation of these indigenous species is not successful, South Africa could lose its floricultural industry to a highly competitive international market (Coetzee *et al.*, 1999).

A large part of the South African population relies on medicinal plants for their primary health care needs and cultural purposes (Grace *et al.*, 2003; Mander, 1999; Cunningham, 1989) and South African traditional medicinal practices have relied on indigenous plants throughout history (Nigro *et al.*, 2004). Traditional medicine is still critical to the primary healthcare needs of rural and semi-urban

livelihood in Southern Africa. Over 700 plant species are actively traded for traditional medicinal uses based on indigenous plants that are generally harvested from the wild (Okole, 2004). An increase in the population needing medicinal plant material has led to severe pressure on the natural resources and significant over-exploitation (Grace *et al.*, 2003; Fennell, 2004; Nigro *et al.*, 2004, Okole; 2004). There are many medicinal plants that also have potential as future horticultural and ornamental plants (de Lange *et al.*, 1989). Amongst these is *Mondia whiteii* (Hook.f.) Skeels, a highly prized, and consequently over-exploited Zulu medicinal plant that is destructively harvested for its roots (McCartan and Crouch, 1998). *Mondia whiteii* is a high climbing vine endemic to the African coastal forests and is distinguished by its strongly aromatic, pleasantly scented rootstock that has a sweet agreeable taste. This species is one of the two focused on in the current work. The aromatic roots are used for both their medicinal and food spice attributes (Koorbanally *et al.*, 2000). Medicinally, the root tissue is reported to relieve stomach-ache, flatulence, abdominal pains, constipation and even bilharzhia. Chemically, rootstocks of *Mondia whiteii* contain ester glycosides with 2-deoxy sugars, small amounts of saponin, a yellow-coloured liquid, and a brown-coloured fixed oil (Koorbanally *et al.*, 2000). The plant stocks and their harvesting have previously not been appropriately managed with little cultivation taking place (McCartan and Crouch, 1998).

While South Africa offers many plant species that are particularly beneficial for medicinal purposes, the floral wealth that exists within the region affords a selection of species that are aesthetically appealing horticulturally, and for outdoor as well as indoor gardening purposes (van Jaarsveld, 1996). One species considered particularly useful for gardening and landscape purposes, the second focus of the present work, is the endemic *Dorotheathus bellidiformis* (Burm.f.) N.E.Br, commonly known as the ice-plant (Burgoyne, 2004). *Dorotheanthus bellidiformis* is a succulent shrub that can be easily distinguished by its brightly coloured flowers, occurring predominantly on sand dunes in the Cape. Despite having aesthetic appeal, this, and other such species have been

used as household remedies for various purposes over the centuries (Pappe, 1847). The expressed juices of the succulent leaves of *D. bellidiformis* have been reported to have antiseptic properties, have long been used for dysentery as a mild diuretic (Pappe, 1847).

Since indigenous plant collectors largely rely on species found in their natural habitats, serious over-exploitation of indigenous plants destined for the herbal trade occurs (de Lange *et al.*, 1989), resulting in a depletion of the natural resources. A combination of high demand, and lack of any meaningful resource management, has resulted in a decline in the supply of numerous indigenous medicinal plants (Mander, 1999). Demands for indigenous plants are rapidly increasing such that natural populations can no longer accommodate these demands (Mander, 1999). Legislation and law enforcement are not practical solutions to the problem of over-exploitation. In order for indigenous plants to sustain the primary health care needs of South Africa, it is necessary for these wild stocks to be supplemented by cultivation. Large-scale cultivation and the production of superior genotypes could greatly ameliorate this problem. Commercial propagation of such plant species will be of great economic benefit to the country (McCartan and van Staden, 1999), reducing the pressure on natural populations and allowing for the continuous availability of plants for traditional healing (van Staden, 1998). An economically viable and lawful trade could thus be initiated and the use of indigenous plants having horticultural, pharmaceutical and timber value could be promoted. However, a large number of the indigenous plant species have only marginal commercial potential as a result of loss of genetic diversity, therefore to realize the full potential of our genetic resources requires long-term breeding programmes (Hannweg, 2004).

### 1.1.3 Crop improvement

Various methods have been established for the propagation of certain indigenous plants. However, there are still numerous other species that can be cultivated commercially if appropriate scientific procedures are ernployed for propagation. Controlled selection and propagation from natural populations has resulted in the current progress of superior horticultural crop species (Abbot and Atkin, 1987; Simmonds and Smartt, 1999). Genetic variation and the selection of superior genotypes from wild populations of plant species have been the essence of plant cultivation practices over the centuries (Lindsey and Jones, 1989). While genetic variation within a plant population is necessary to isolate individuals for cultivation (Simmonds and Smartt, 1999), the isolation of superior genotypes from natural sources is difficult because of the rapid reduction in genetic variability that has occurred in natural populations. As a result, crop improvement strategies have to be employed to introduce genetic variability artificially in species that are commercially important or have potential for commercialization.

According to Simmonds and Smartt (1999), the genetics of crop plant species has been a vital constituent of plant breeding programmes throughout history although propagation methods can be implemented to increase the supply of plants, because of the reduction in the genetic variation within the populations, the isolation of superior genotypes for commercial purposes is practically impossible. Thus significant genetic manipulation of plants needs to be carried out to develop new varieties from which selection for commercial purposes can take place (Simmonds and Smartt, 1999).

The primary aim of the breeder is to improve plants by bringing about favourable combinations of genes (Broertjes and van Harten, 1988). The genetic variation within a population of a particular plant species is controlled by differences in the kind, structure and amount of genetic variation, the source of these sudden genetic changes is referred to as mutations (Broertjes and van Harten, 1988).

Improvement of plant species for commercial purposes has progressed from collection, selection and cultivation of wild plants to more scientifically based plant breeding techniques that can accelerate the production and release of new and better plant varieties (Abbot and Atkin, 1987 and Lindsey and Jones, 1989). The use of mutation breeding and tissue culture techniques are advances that have been particularly useful in crop improvement programmes for plant species which are propagated vegetatively (Abbot and Atkin, 1989; Lindsey and Jones, 1989). A mutational change that has been particularly useful in several horticultural plant species has been a change in chromosome number, most importantly, an increase in chromosome number from the 'conventional' diploid, to a polyploid status (Cohen and Yao, 1996; Väinölä, 2000; Väinölä, 2001; Shao *et al.*, 2003; Thao *et al.*, 2003 and Eeckhuant *et al.*, 2004). Most early published literature concerning mutational breeding seemed to have excluded changes in ploidy level as a technique of mutational breeding (Broertjes and van Harten, 1988). Practically however, the outcome of changes in ploidy status is important in creating genetic variation and therefore is considered a method of induced mutations by Broertjes and van Harten (1988).

## 1.2 LITERATURE REVIEW

### 1.2.1 Polyploidy

Increase in the chromosome number from the common diploid number is referred to as polyploidy (Broertjes and van Harten, 1988; Lee and Chen, 2001) and, is a feature of angiosperm evolution (Soltis and Soltis, 1995; Jiang *et al.*, 1998; Nelson and Elison, 1999; Husband, 1999; Martinez-Perez *et al.*, 2000; Otto and Whitton, 2000). Eighty percent of the worlds' angiosperms are polyploids (Song and Osborn, 1994; Song *et al.*, 1995; Soltis and Soltis, 2000). Polyploidy has been considered one of the key features that has allowed angiosperms to colonize various habitats successfully (Soltis and Soltis, 2000; Ha *et al.*, 1999; Li *et al.*, 1996 and Soltis and Soltis, 1995) and is also considered the means by

which certain species have evolved particularly high chromosome numbers. According to Soltis and Soltis (1995), polyploids are traditionally viewed as possessing fundamentally different characteristics than diploids (Soltis and Soltis, 1995), while differences between natural polyploids and synthetic polyploids were reported to be insignificant (Ozkan *et al.*, 2001).

Examples of natural polyploids include some of the world's most economically important plants such as wheat, soybean, potatoes, sugarcane and cotton (Hilu, 1993; Song *et al.*, 1995 and Wendel, 2000). The use of tetraploid *Gossypium* (cotton) varieties in breeding programmes has been highly profitable, offering superior quality relative to some diploid varieties (Jiang, 1998). Although natural polyploids have gained tremendous success as agricultural crops, a number of induced polyploid cultivars have been incorporated successfully, e.g. in active citrus breeding programmes (Soost, 1987; Wu and Mooney, 2002). The genus, *Lilium*, is an important horticultural crop with numerous commercially grown cultivars in production worldwide; however, continuous research is being carried out to introduce potentially new induced polyploid cultivars commercially (Broertjes and van Harten, 1988 and van Tuyl *et al.*, 1992).

*1.2.1.1 Changes associated with increased ploidy level*

Although many changes have been reported with an increase in ploidy level, there are no universal patterns associated with such change (Levin, 1983 and Lee and Chen, 2001). However, a few patterns that are commonly encountered with an alteration of ploidy level include: changes in the genetic system; changes in cell size (Levin, 1983); and a loss of fertility (Lyrene and Perry, 1982). There are many aspects of the genetic system of polyploids that have contributed to their success (Levin, 1983; Husband, 1999 and Soltis and Soltis, 2000). An increase in chromosome number causes epigenetic gene inactivation (Scheid *et al.*, 1996; Comai, 2000; Lee and Chen, 2001), creating differences in gene expression patterns (Scheid *et al.*, 1996). The frequency of such changes is

faster than that of mutations and is transferred from one cell to its progeny in a mode similar to the transfer of genes that have undergone mutations (Scheid *et al.*, 1996). Epigenetic changes can effect many genes simultaneously, or only a specific gene (Comai, 2000 and Lee and Chen, 2001). The genetic re-organization a polyploid population undergoes creates possibilities for alleles favoured in the diploid background to be replaced by those favoured in the polyploid background (Levin, 1983).

The immediate effect of an increase in chromosome number in plants is an increase in cell size (Byrne *et al.*, 1981 and Levin, 1983). This alters the geometric relationship between the nucleus and the rest of the cell, which in turn is thought to affect the metabolic rate and growth rate, processes that are dependent on this relationship (Levin, 1983). An alteration of cell geometry and an increase in chromosome number results in a breakdown of balanced systems of gene interactions (Levin, 1983; Sotis and Soltis, 1999; Comai, 2000). Changes in cell geometry result in changes in the ratio of the area of the nuclear membrane to chromatin volume. More chromatin is in contact with the nuclear membrane and a greater proportion of chromatin is in condensed regions along the nuclear membrane. Lowering the ratio of the nuclear membrane to chromatin volume and changes in the chromatin-membrane relationship can alter gene expression and regulation. The pattern of telomere association during interphase is also altered during polyploidization (Vega and Feldman, 1998 and Martinez-Perez *et al.*, 2000), which also alters gene expression (Ashley and Wagenaar, 1974; Fussel, 1975 and Avivi and Feldman, 1980). Changes in surface area-to-volume ratio of cells through nuclear enlargement has been reported to have cascading physiological and morphological effect as membrane sites become limiting and cellular concentrations change (Levin, 1983). Changes in cell size also results in changes in the size of plant organs such as flowers (Eeckhaunt *et al.*, 2004)

The percentage of DNA coding for ribosomal RNA varies widely within species. Cullis and Davies (1974) found an absence of differential amplification of the RNA cistron with different ploidal levels in investigations of n, 2n, 3n, 4n and 6n *Datura innoxia*. Similarly Ingle *et al.* (1976) working with 2n, 3n and 4n varieties of *Hyacinthis orientalis* showed that the RNA content was the same in all. The product level remained constant even when nucleolar organizing regions were varied through polyploidy. This showed that RNA content is not regulated by specific gene dosage. However, Tal (1977) showed that polyploidization usually increases enzyme activity per mg protein. Polyploidization does not affect all enzymes in the same way, therefore leading to new favourable or altered negative balances in metabolic regulation.

Polyploidy can affect the structure of some enzymes (Levin, 1983). Two reasons for this change include sequence elimination, which occurs very early during polyploidization and homologous recombination (Song and Osborn, 1994). Homologous recombination occurs between repeated regions; this destabilizes the genome by producing deletions and chromosome rearrangements. Closely-related species that differ in ploidy level often differ in life-history and, in some cases morphology (Thompson *et al.*, 1997).

Polyploidization has an effect on the regulatory influence of plant growth regulators (PGRs). Diploids have been shown to have higher PGR levels than polyploids. In many species carbon dioxide exchange rate changes as ploidy levels change, as was shown in studies by Byrne *et al.* (1981) working with *Festuca arundinacea*, where higher $CO_2$ exchange rate was expressed as ploidy level increased. Polyploidization can alter the secondary biochemistry of a plant in a qualitative manner. Tissue specificity of secondary compounds is altered with changes in ploidal levels. Differences in longevity have been found between diploid and tetraploid plants and a shift from annual to perennial habit can also occur. Reduced growth may accompany polyploidization. However, comparative studies of colchicine-induced polyploid plants showed that tetraploid plants grew

more vigorously than diploids (Gao *et al.*, 1996). Net photosynthetic rates were reported to be higher in tetraploid perennial ryegrass genotypes than in the diploid genotypes (Rathnam and Chollet, 1980 and Schapendonk *et al.*, 1990). Li *et al.* (1996) showed that polyploid *Betula papyrifera* genotypes are more tolerant to water deficit than their diploid relatives. Polyploid clones were shown to express vigorous juvenile growth making them more suitable for short rotations at dense spacing relative to diploid clones (Borzon *et al.*, 1994). Autotetraploid plants of a cultivated tomato variety were reported to have a higher water content than the diploid counterpart (Tal and Gardi, 1976). The net gas exchange rate observed in autotetraploid citrus was reduced compared with its diploid relatives (Romero-Aranda *et al.*, 1997). Herbage growth and carbohydrate metabolism reported in tetraploid alfalfa genotypes was greater than in the diploid genotypes (Volnec, 1988).

Polyploidization alters floral mechanism and life cycle, hence altering the relationship between flowers and their pollinators, and Cohen and Yao (1996) reported that pollen grains from polyploid *Zanthedeschia* cultivars were larger than those from diploid plants. Growth of tetraploid embryos and endosperm tissue is faster than in diploids. Larger seed sizes have been recorded for tetraploid species, making tetraploid seedlings more vigorous and rapid-growing than the diploid counterparts. Schifino and Moreas-Fernandes (1987) showed that field production of seeds was high in induced polyploid *Trifolium riogranadense*.

Some studies have shown that some polyploids are more resistant to pathogens, insects and nematodes than closely related diploids, and polyploidy can therefore be considered a means by which plant species can create a barrier to biological attack (Burdon and Marshall, 1981; Thompson *et al.*, 1997). Some polyploids are more tolerant to drought than their diploid counterparts (Sivolapov and Blagodarvo, 1994). A comparative study of *Betula papyrifera* of different ploidal levels by Li *et al.* (1996) showed that the polyploid population had higher water

use efficiency than the diploid population, and could survive higher periods of water deficits. This was accounted for by differences in leaf morphology, leaf anatomy, water relations, gas exchange and growth characteristics. Wachira and Ng'etich (1999) showed that differences in ploidy level effect the dry matter productivity of tea (*Camellia sinensis*). Differences in growth rate in different ploidy levels of perennial ryegrass (*Lolium perenne*) were reported by Schapendonk *et al.* (1990). Polyploidy offers resistance to harsh climatic conditions, but the response direction varies between species. It is also thought that polyploidy influences the temperature optima for various physiological processes (Levin, 1983).

Induced polyploidy has been shown to be most useful in producing genetic bridges in agronomic and floricultural crop (Hancock, 1997). Griesbach (1985) reported that induced polyploidy can play an important role in the improvement of *Phalaenopsis* orchids. Differences were recorded in fruit and plant morphology of induced polyploids of strawberry by Predieri *et al.* (1989). A synthetic tetraploid *Eustomia grandiflorum* ("Blue poppy") had thicker stems and reduced height in comparison to diploid plants (Griesbach and Bhat, 1990). These characteristics were horticulturally favoured since the stems were able to support the flowers for longer without bending. Induced tetraploid azaleas showed a general increase in size and firmness of flowers (Pryor and Franzier, 1968). Cytological investigations by Schifino and Moreas-Fernandes (1987) of polyploid *Trifolium riogranadense* an important forage crop, showed that the polyploid variety had good prospects for utilization and breeding.

Apart from an increase in cell size, a universal and undesirable effect of increasing chromosome number, is a decrease in fertility (Levin, 1983). The presence of additional chromosomes possessing a similar structure can create a disruption of chromosome pairing during mitoses, resulting in the production of non viable, less functional gametes thereby leading to infertility (Martinez-Perez *et al.*, 2000). In order to produce viable gametes, polyploids must "behave

effectively" as diploids during meiosis. Crops that are most amenable to polyploidization should have a low chromosome number, be harvested primarily for their vegetative parts and be cross-pollinating (Thompson *et al.*, 1997). The perennial habit and vegetative reproduction have bearing on polyploid success (Thompson *et al.*, 1997).

Polyploid breeding is most feasible for crops that are grown primarily for their vegetative parts. These crops benefit from one universal consequence of polyploidy i.e. larger plant parts (Lyrene and Perry, 1982). A broad genetic base, including polyploidy is essential for any breeding programme. According to Hancock (1997), inadequate variation in the original breeding population has undoubtedly contributed to the failure of several polyploid breeding programmes. Artificially induced polyploids suffer meiotic irregularities, and although this is not a problem for flower, root and leaf crops, such reduced fertility can affect seed production and fruit development, which is adverse for crops that are primarily grown for their seeds and fruits. Other problems associated with induced polyploidy include inappropriate changes in nuclear architecture, slowed development rates and poor adaptation (Bennett, 1972; Levin, 1983).

Fertility and seed yield in most medicinal plants is not as important as that in crop plants, because in most cases, leaf, stem and root parts are used for medicinal purposes (Gao *et al*, 1996). However, the content of medicinally active compounds in those various plant organs is very important. For these reasons various investigations have been carried out to evaluate the effectiveness of polyploidization on yield and production of secondary metabolites in medicinal plants (Chavdej and Becker, 1984; Gao *et al.*, 1996). Gao *et al.* (1996) investigated the effects of induced polyploidy on *Salvia miltiorrhiza*, a traditional Chinese medicinal plant. The roots are used as an important drug to cure cardiovascular disease. Although all tetraploid plants produced were semisterile, higher contents of the effective compounds were obtained in roots of the tetraploid plant (Gao *et al.*, 1996).

The intention of breeding induced polyploids is to capitalize on the direct consequences of polyploidization i.e. larger cells and plant parts. Polyploidy has been used as a breeding tool in horticulture for obtaining new ornamental characteristics (Shao *et al.*, 2003). In addition to these direct uses, induced polyploidy may be used to facilitate genetic transfer between taxa thus serving as an agent of introgression. It is apparent that different types of crop respond differently to induced polyploidy. Original ploidy level, genome structure, mode of reproduction, perenniality and plant part for which the crop is grown all have bearing on the breeding success or failure. The plant breeder must match the characteristics of the plant to the specific application of induced polyploidy that maximizes the opportunity for success (Soost, 1987). Breeders should carefully weigh the relative merits of polyploid breeding against other breeding techniques and commit resources to the programme that offers the greatest potential (Awoleya *et al.*, 1996; Hancock, 1997). Often methods comprising both conventional, and polyploid breeding methods are appropriate (Awoleya *et al.*, 1996).

Polyploids are generally stockier than the diploids they are derived from, because they have larger cells. Leaves are thicker, larger and greener. Roots, flowers and seeds also show an increase in size (Griesbach, 1990; Gao *et al.*, 1996; Takamura and Miyajima, 1996; Väinolä, 2000, Väinolä, 2001; Shao *et al.*, 2003). Although these are the properties most commonly associated with changes in ploidy level, polyploids can also differ in vigour compared with their diploid parents. Different genotypes within a species respond differently to induced polyploidy (Predieri *et al.*, 1989). The performance of a genotype as a diploid is a poor indication of its performance after it has been converted into a polyploid. In order to identify a superior genotype at the polyploid level, a large number of genotypes need to be converted to polyploids and new breeding programmes need to be initiated with the polyploids (Awoleya *et al.*, 1996). In order to induce polyploidy in a large number of genotypes it is important to use seed-derived material for polyploid induction treatments.

*1.2.1.2 Methods to induce polyploidy in plants*

Polyploidy can be obtained using unreduced gametes (Griesbach, 1985), environmental shock treatment, somatic chromosome doubling through tissue culture of various explants (Chauvin *et al.*, 2003) or treating plant material with chemicals that disrupt normal chromosomal division. Polyploids were also reported to be produced from variant nucellar seedlings from several citrus cultivars (Soost, 1987). Several chemicals can induce polyploidy. The most frequently used chemical has been colchicine (Griesbach, 1985; Hancock, 1997). This alkaloid is extracted from seeds or corms of the Autumn crocus (*Colchicum autumnale*) (Hancock; 1997). Colcemid, a synthetic equivalent, has also been used. Colchicine was used to induce chromosomal doubling during the 1900s (Hancock, 1997). It binds to tubulin (a hetrodimer of microtubules) and prevents the assembly of these hetrodimers into microtubule (Artvinli, 1987). Survival of the treated plant material depends on the concentration of the antimitotic substance used and the genotype that is being polyploidized (Predieri *et al.*, 1989).

The assembly of microtubules is essential for spindle formation during cell division (Dawe, 1998; Gunning and Hardham, 1982; Pickett-Heaps, 1974) and are implicated in several motility-related processes during the mitotic cycle and differentiation, including chromosome migration, and afford the tracks for vesicle transport and the transport and orientation of cellulose microfibril deposition in the developing cell wall (Gunning and Hardham, 1982). Since, microtubules are associated with separation of chromosomes to different regions of the cell, blocking the binding sites of microtubles ultimately results in the inhibition of spindle formation at the point of separation of the daughter chromosomes to opposite poles of the cell. This results in a polyploid cell forming. Colchicine is effective when applied to germinating seeds (Sanders and Hull, 1970), young seedlings (Schifino and Moraes-Fernandes, 1987), roots (Taira *et al.*, 1991) or to growing points such as shoots or buds (Lyrene and Perry, 1982).

The method of colchicine application plays a role in the frequency of polyploidy production. Several methods of colchicine application have been evaluated. These include: immersion of whole plants, cuttings or organs such as roots in a solution of colchicine (Taira *et al.*, 1991; Schifino and Moraes-Fernandes, 1987; van Tuyl *et al.*, 1992), application of the solution to the meristematic region with a syringe or medicine dropper (North, 1976; Barett, 1974) or with cotton-wool soaked in a colchicine solution, and applying the solution to the growing region with a fine brush. However in most published literature, it was shown that the most effective method used, was shown to be the immersion of plant tissue into a solution of the antimitotic agent for several hours, followed by several rinses with distilled water.

Although immersion of plant material in a solution of colchicine *in vivo* can produce high frequencies of polyploids relative to other methods of application, some investigators have shown that *in vitro* colchicine treatment produce polyploids with higher efficiency (Cohen and Yao, 1996). Success is achieved because *in vitro* produced shoots are far easier to manipulate than greenhouse-maintained plants. The meristems of *in vitro* shoots are composed of a lower number of cells than those of *in vivo* plants and therefore the chances of obtaining non-chimeral mutants will be greater (Predieri *et al.*, 1989).

*1.2.1.3 Advantages of in vitro treatment with antimitotic substances*

Gao *et al.* (1996) highlighted several advantages of inducing polyploidy *in vitro*.
A large number of plants can be treated effectively and accurately in tissue culture by adding colchicine to prepared media. Determining chromosome number in tissues of polyploid plants produced *in vitro* is more convenient and effective in comparison to field-grown plants. *In vitro* polyploidization was reported to increase efficiency and decrease occurrence of chimeras than *in vivo* polyploidization. Selected polyploid plant material can be rapidly regenerated via tissue culture. It is easier to use different methods to polyploidize plants with

colchicine *in vitro*. Plant material can be cultured on a solid medium containing an antimitotic substance for a length of time and thereafter cultured on medium free of colchicine (Cohen and Yao, 1996; Lyrene and Perry, 1982) or the material can be immersed in liquid medium containing a antimitotic substance (Lyrene and Perry, 1982), rinsed several times with sterile distilled water and cultured on medium lacking the antimitotic substance. Lyrene and Perry (1982) showed that higher frequency of polyploid individuals can be achieved when plant material is treated with colchicine in a liquid medium than in a solid medium. Once polyploidy is identified following treatment, rapid *in vitro* multiplication of the polyploid tissue is possible (Lyrene and Perry, 1982).

Various explant cultures can be treated with colchicine under *in vitro* conditions. These include: whole seedlings, plant organs such as bulbs or corms (Griesbach, 1989), cuttings (Lyrene and Perry, 1982; Awoleye *et al.*, 1996), somatic embryos (Kato, 1989), cell suspension cultures (Hienz and Mee, 1970; Chavadej and Becker, 1984; Dolezel *et al.*, 1989), callus cultures (Hassawi and Liang, 1997) or *in vitro* propagated shoots (Priederie *et. al.*, 1989; Cohen and Yao, 1996). As a general rule, the choice of explant depends on the species to be polyploidized, and the most favourable means of *in vitro* propagation. There is extensive published literature featuring the effectiveness of *in vitro* polyploidization treatments and the implications of the choice of explant used (Table 1.1).

**Table 1.1:** Some successful *in vitro* polyploidization studies carried out on plant species particularly important for their horticultural and medicinal properties.

| Species | Explant | Reference |
|---|---|---|
| *Punica granatum* (Pomegranate) | Shoots propagated *in vitro* | Shao *et al.*, 2003 |
| *Solanum spp* | Apical buds | Chauvin *et al.*, 2003 |
| *Miscanthus sinensis* | *In vitro* plantlets, *in vitro* shoots<br>Callus | Peterson *et al.*, 2003 |
| *Spathiphyllum wallisii Regel* | Somatic embryos | Eeckhaunt *et al.*, 2004 |
| *Zanthedeschia* cultivars | Shoot cultures | Cohen and Yao, 1996 |
| *Solanum tuberosum*<br>*Solanum circaeifolium* | Shoot nodes | Demaine and Simpson, 1999 |
| *Medicago sativa*<br>*Medicago varia* | Cell suspension cultures | Dolezel *et al.*, 1989 |
| *Salvia miltiorrihizia Bge* | Bud cultures | Goa *et al.*, 1996 |
| *Rhododendron simssi* | Seedlings | Eeckhaunt *et al.*, 2002 |
| *Allium cepa L.* | *In vitro* propagated plantlets | Geoffriau *et al.*, 1997 |
| *Vaccinium ashei Reade*<br>*Vaccinium alliotti Chapm* | *In vitro* shoots | Lyrene and Perry, 1982 |
| *Camellia japonica* L. | Somatic embryos | Kato, 1989 |
| *Sacharum officinarum* L | Cell suspension cultures | Heinz and Mee, 1970 |
| *Citrus* | Somatic embryogenic callus | Wu and Mooney, 2002 |
| *Valeriana wallichi* DC | Cell cultures | Chavadej and Becker, 1984 |
| *Alocasia* | Shoot tips | Thao *et al.*, 2003 |
| *Fragaria* x *ananassa* Duch | *In vitro* shoots | Predieri *et al.*, 1989 |
| *Actinidia* | Stem segments, seeds, seedlings and petioles | Fraser *et al.*, 1992 |

### *1.2.1.4 Factors influencing in vitro polyploidization*

There are various factors that affect *in vitro* polyploidization (Sanders and Hull, 1970; Schifino and Moraes-Fernandes, 1987; Taira *et al.*, 1991), one being the pH of the colchicine solution used, which has been reported to have an effect on induced polyploidy (Neubauer and Thomas, 1966; Taira *et al.*, 1991). Environmental conditions under which the treated plants are maintained

influence the frequency of polyploid formation, and higher polyploid frequency has been reported to occur under controlled growth conditions where temperature and light are regulated (Taira *et al.*, 1991). This is also a possible explanation for the high success rate of *in vitro* chromosome doubling that has been reported in several investigations e.g. in those reported by Predieri *et al.* (1989) or Cohen and Yao (1996) Polyploid formation is also genotypically dependent (Peterson *et al.*, 2003). Several reports have shown that different cultivars and varieties respond differently to colchicine treatment (Barret, 1974; Predieri *et al.* 1989; Hassawi and Liang, 1991 and Talukdar and Sen, 1997).

Mutational changes that arise after chromosome doubling have been shown to be heritable (Hassan *et al.*, 1991). Since a significant factor influencing successful polyploidization treatment is the colchicine concentration used, many investigations have examined the effect of colchicine concentration on polyploidization *in vitro* and *in vivo*. Duration of colchicine treatment, together with colchicine concentration, has been reported to be significant factors in the efficiency of polyploidy formation. In a lily breeding programme, treatment with a 0.05 – 0.1% colchicine solution was reported to be most effective in inducing polyploidy, while a 0.2% colchicine solution was reported to be most effective in polyploidization of orchids (Griesbach, 1985). *In vitro* treatment with colchicine for 24 – 48 hours has been most effective in several investigations (Lyrene and Perry, 1982; Dolezel *et al.*, 1989; Hassawi and Liang, 1991; Awoleye *et al.*, 1996; Geoffriau *et al.*, 1997).

Colchicine, has in the past, provided a tool for key discoveries on the dynamics of chromosomal inheritance and evolutionary relationships of many crop taxa (Gunning and Hardham, 1982; Hancock, 1997 and Eckardt, 2001), its direct horticultural importance in respect to induced polyploidy has been limited outside the floricutural industry (Hancock, 1997). Colchicine is carcinogenic and binds with high affinity to tubulin in mammalian cells, inducing metaphase arrest in animal cells at $10^{-7}$M concentrations (Morejohn *et al.*, 1987). It has also produced

undesirable mutagenic activity in plants (van Tuyl *et al.*, 1992; Thao *et al.*, 2003). Sensitivity of higher plant cells to colchicine is less than that of animals by one to three orders of magnitude, therefore the concentrations necessary for metaphase arrest in plant cells are significantly higher, which is attributed to the lower binding affinity of colchicine to plant tubulin (Morejohn and Fosket, 1984; Morejohn *et al.*, 1987). Therefore high concentrations, that are often toxic to humans and other animals, are used to induce polyploidy in plants (Morejohn *et al.*, 1987). Responses of plants to colchicine vary, and where present, may occur at concentrations so high as to bring about non-specific effects. As with other chemically-induced perturbations, colchicine can affect different catagories of plant microtubule activity differentially even within the same cell (Levin, 1983).

Other mitotic inhibitors that are effective at much lower concentrations than colchicine have therefore been evaluated to induce chromosome doubling in plants. One such chemical is oryzalin (3,5-dinitro-$N^4$, N-dipropylsulphanilamide), a dinitroaniline herbicide (Morejohn *et al.*, 1987). Dinitroanalines comprise a large class of herbicides that causes gross morphological changes in plants, particularly in regions of high meristematic activity. Oryzalin was reported to mimic the symptoms of colchicine but are active at 1000-fold lower concentration and was found to associate strongly with plant cell membranes. Oryzalin has been shown to bind to plant tubulin with greater affinity than colchicine (Verhoeven *et al.*, 1990). Histological studies of dinitroanaline-treated roots have shown that cell division and mitosis are disrupted after brief exposure, and cells in the meristematic regions become swollen. Effected cells contain a polyploid chromosome number and do not form cell plates following nuclear division. These gross changes in morphology and cytological features are similar to the effects of exposure to colchicine (Morejohn *et al.*, 1987; Verhoeven *et al.*, 1990; Peterson *et al.*, 2003).

The chemical structure of oryzalin and colchicine are remarkably different from each other (Figure 1.1). Morejohn *et al.* (1987) provide evidence that oryzalin

directly inhibits the dynamics of microtubule assembly and disassembly in higher plants. Several investigators have shown that considerably lower concentrations of oryzalin, relative to colchicine concentrations, are adequate to induce chromosome doubling in plants. Geoffriau *et al.* (1997) showed that 50 μM oryzalin and 2.5 mM colchicine induced polyploidy in gynogenic clones of onion (*Allium cepa*). Although colchicine and oryzalin were both found to affect plant regeneration, better plant quality was found to occur with oryzalin (50 μM). van Tuyl *et al.* (1992) reported that *in vitro* treatment of *Nerine* and *Lillium* with concentrations varying from 0.001% - 0.01% appeared to be less inhibiting for regeneration and resulted in a higher number of polyploid plants surviving than treatments with colchicine, where a tenfold higher concentration was necessary.

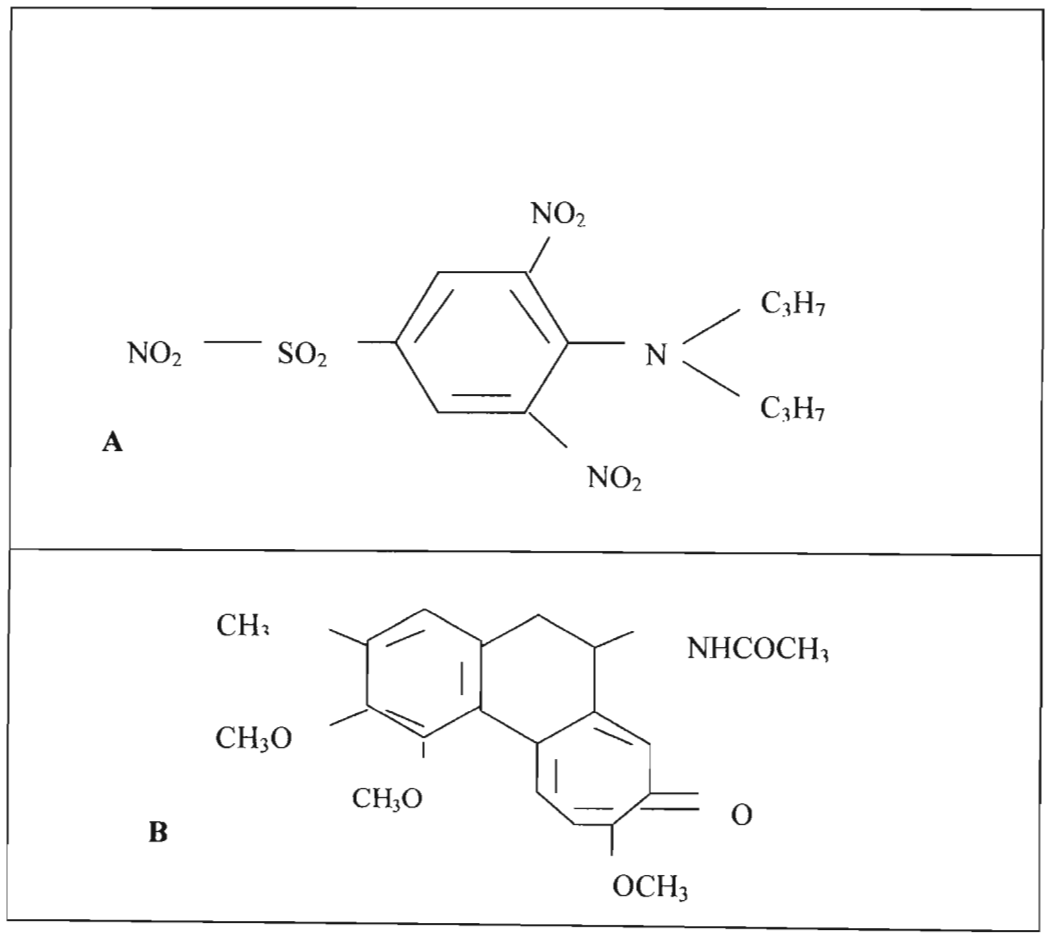


**Figure 1.1:** Structure of antimitotic compounds: A – oryzalin; B- colchicine

While *in vitro* polyploidization is considered a far superior and convenient method of inducing polyploidy than conventional methods that have been previously used (Cohen and Yao, 1996; Wu and Mooney, 2002; Shao *et al.*, 2003). While the technology of *in vitro* plant tissue culture provides favourable opportunities for a plant breeder to manipulate chromosome number *in vitro*, plant tissue culture encompasses a range of techniques that are necessary to understand so that successful culture of plant material *in vitro* is possible. Practical constraints associated with *in vitro* plant culture systems (Murashige, 1977) need to be identified and minimized when inducing polyploidy *in vitro*, as these constraints will ultimately restrict the survival and response of plant material to *in vitro* antimitotic treatments.

### 1.2.2 *In vitro* plant tissue culture

#### *1.2.2.1 In vitro plant propagation*

Plant tissue culture can be defined as a laboratory procedure that facilitates the growing of "sterile" plant cells or tissues separate from the mother plant on artificial media under controlled conditions in small culture vessels in which all the nutrients necessary for growth are provided (Wetherell, 1982; Torres, 1989; George, 1993 and Leifert and Waites, 1990). The possibility of maintaining metabolically active plant cells under *in vitro* conditions has offered scientists an important method that has permitted the study of plant metabolism, genetics, morphogenesis and plant physiology, plant tissue culture has also been important for the genetic manipulation of plants (for example polyploidization) (Murashige, 1977), the elimination of plant pathogens, the preservation of important plant species in limited space (Engelmann, 1997) and in the multiplication of plant tissues *in vitro* (Leifert and Waites, 1990).

Techniques of plant tissue, cell an organ culture have become well established in research laboratories worldwide (George, 1993). Various procedures for the

tissue culture of a range of plant species have been developed (Refer to Table 1.2) for the generation and screening of desirable variants, cellular cloning and rapid propagation of genotypes (Murashige, 1977 and George, 1993), induction of haploid tissue from anther and pollen cultures (Murashige, 1977), extending the range of genetic variability through induced mutations and somatic clones (Broertjes and van Harten, 1988 and Lindsey and Jones, 1989), and the formation of isolated callus and cell cultures for studies on the effects of nutrients, vitamins and plant growth regulators (Murashige and Skoog, 1962) on cell growth and differentiation (Murashige and Skoog, 1962). Plant tissue culture has also become particularly useful for the propagation of superior plant genotypes commercially (Torres, 1989; Hartman *et al.*, 1997), since appropriate conditions can be achieved to permit the development of individual plants from a small piece of the mother plant that is genotypically identical to the parent plant. Plant tissue that has been genetically altered can also be manipulated *in vitro* to facilitate the perpetuation of genetically altered plants.

*1.2.2.2 Plant propagation*

Propagation refers to the controlled multiplication of plants from seeds or organs i.e. either sexually or asexually (George, 1993; Hartman *et al.*, 1997). Sexual reproduction involves the development of an embryo that is contained within a seed that has been obtained from the union of gametes (Hartman *et al.*, 1997), therefore the resultant seedlings will be genetically diverse, representing new combinations of genes (George, 1993). Seeds are often produced in large numbers and easily distributed therefore plants regenerated from them are individually inexpensive, while seeds of many species can be stored for long periods without loss of vigour or viability. However, for many agricultural and horticultural purposes, it is desirable to cultivate clones which are genetically identical (George, 1993). Vegetative propagation is the only means to achieve this, thereby perpetuating genotypes and multiplying plants displaying unique and desirable characteristics.

Asexual reproduction (vegetative reproduction) involves an increase in plant number through somatic cell division and differentiation, thus ensuring that the unique characteristics identified in the parent are passed to the new plants (Sirnmonds and Smartt, 1999). An important aim of such techniques is to multiply superior genotypes in sufficient quantities (Gamborg and Shyluk, 1981). Many plant species can be easily propagated vegetatively from cuttings (George, 1993).

Traditional methods for vegetative propagation involve the use of rooted cuttings (Gamborg and Shyluk, 1981), while for bulb species, techniques including stem scaling, basal and bulb cuttings are used (McCartan and van Staden, 1999). Rooted cuttings can produce a single plant which can provide material for future cuttings, several years later. However many plants propagated by rooted cuttings are characterised by a rapid loss in rooting efficiency with increasing age of the parent plant (Gamborg and Shyluk, 1981). In contrast, even the most limited *in vitro* shoot culture system used today can produce several axillary buds and thus axillary shoots that can in turn produce additional shoots (Hartman *et al.*, 1997). Subsequent rooting produces many individual plants, so alleviating the problem of the lack of seeds, poor seed germination and slow vegetative multiplication. Thus micropropagation has been chosen as a preferred means of multiplication of several indigenous plants (de Lange *et al.*, 1989). The rapid multiplication rates achieved through plant propagation by tissue culture is an advantage, and thus *in vitro* propagation is considered an invaluable tool to rapidly produce large quantities of uniform plants with superior characteristics (Gamborg and Shyluk, 1981).

While asexual multiplication of plants using tissue culture techniques can be achieved by the multiplication of shoots from axillary buds (Torres, 1989; George, 1993), other means include the formation of adventitious shoots or adventitious somatic embryos directly on pieces of explants, or indirectly by the proliferation of cells within the explant leading to semi-organised callus tissue (George, 1993).

Somatic embryogenesis results in the formation of a bipolar embryo through steps that are apparently similar to zygotic embryo formation (Hartman *et al.*, 1997).

While the application of plant tissue culture offers a variety of opportunities for commercial and research laboratories, the basic principles of procedures are consistent across all borders and these principles need to be well understood before any tissue culture procedures are carried out (Torres, 1989). There are basically four stages associated with micropropagation in order for the procedure to be used for commercial propagation, namely sterilization and establishment of explants in culture, shoot multiplication, root formation and acclimatization (Wetherell, 1982; Torres, 1989; George, 1993; Hartman *et al.*, 1997). The requirements for each stage vary according to the source material and propagation method, thus these stages may not always fit neatly into compartments (Torres, 1989; George, 1993).

*1.2.2.3 Aspects of in vitro plant tissue*

A plant tissue culture is initiated from a small piece of a plant (parent plant), known as the explant (George, 1993). The part of the plant from which the explant is obtained depends on the kind of culture to be initiated, the purpose of the culture and the plant species to be used (Gamborg and Shyluk, 1981; Wetherall, 1982; Torres, 1989). The choice of explant used has important consequences on the success or failure of tissue culture (George, 1993). Source plants obtained from the field, or even greenhouse, are seldom free of contaminants, which must be eliminated from explants taken for tissue culture. Nevertheless numerous contaminants cause problems in plant tissue culture, and these include viruses, bacteria, yeasts, fungi, mites and thrips (Leifert and Waites, 1990; Hol and van der Linde, 1992; George, 1993; Leifert and Cassells, 2001; Thomas, 2004). The media on which the explants are grown provide ideal conditions for the growth of the micro-organisms (Thomas, 2004), which compete

adversely with plant material growing *in vitro* (Kunneman and Faaij-Groenen, 1988) and, if pathogenic destroy the explant. It is thus necessary for plant cultures to be established and maintained in aseptic conditions and the control of internally-borne pathogens is also vital. Therefore the initial procedures to establish an *in vitro* plant culture require the removal of all culturable fungal and bacterial contaminants (Niedz and Bausher, 2002) and it is also necessary to sterilize tools used for dissection as well as the vessels and media in which the cultures are grown (Wetherell, 1982; George, 1993; Torres, 1989). Sterilization includes the use of chemicals that are toxic to micro-organisms but non-toxic to plant material, including antibiotics and fungicides, alcohols, mercuric chloride, and oxidizing biocides such as halogen compounds or hydrogen peroxide (Reed and Tanprasert, 1995; Niedz and Bausher, 2002). One of the most common sterilants used in plant tissue culture is chlorine in the organic form of calcium or sodium hypochlorite (Niedz and Bausher, 2002). The halogen compound sodium hypochlorite, which is readily available commercially as household bleach under various trade names, is probably the most commonly used sterilant (Hartman *et al.*, 1997). A primary disinfectant is ethyl alcohol, which can be used prior to treatment with bleach (Niedz and Bausher, 2002; Torres, 1989).

Bleach is diluted with sterile distilled water before being used. A few drops of a surfactant or detergent are added to the bleach solution to improve surface contact with the explant (Torres, 1989). The effectiveness of a bleach treatment is a time-dosage response (Wetherell, 1982; George, 1993; Hartman *et al.*, 1997), increasing with both time and concentration, but damage to the living tissue also increases, therefore a compromise has to be developed through vigorous testing for each explant-type to be disinfected (Wetherell, 1982). After a predetermined immersion time in the disinfecting solution, the solution is decanted and the explant is rinsed at least three times with sterile distilled water (Wetherell, 1982; Torres, 1989).

Once the explant is sterilized it is placed on sterile nutrient medium (Torres, 1989; George, 1993). Plant material can grow *in vitro* only if it is supplied with a specialized medium (Torres, 1989; Gamborg and Shyluk, 1981; George, 1993).

An important factor that governs the growth and morphogenesis of plant tissues in culture is the composition of the culture medium. A medium commonly consists of a solution of salts supplying the major and minor elements necessary for growth of whole plants together with various vitamins, amino acids and a carbon-rich energy source which is usually in the form of sucrose (Murashige and Skoog, 1962; Gamborg amd Shyluk, 1981; Torres, 1989; George, 1993). Plant material can be cultured in liquid medium or on partially solidified medium (Torres, 1989 and George, 1993). Media which are partially solidified incorporate a gelling agent, usually agar. Semi-solid media are used for explant establishment, for routine culture of callus or plant organs in micropropagation and long-term maintenance of plant cultures (Torres, 1989).

The disinfected explant is cultured aseptically in a medium that will confirm its freedom from micro-organisms, as well as allowing it to initiate growth (Wetherell, 1982; George, 1993). Any explants or media showing contamination is discarded. The establishment of explants usually requires about 4-6 weeks to complete in order to produce plants ready to transplant to multiplication medium (Wetherell, 1982; Torres, 1989).

Once an explant is established within culture it is transferred onto a multiplication medium (George, 1993 and Hartman *et al.*, 1997). The main objective at this stage is the proliferation to produce outgrowths that, when separated, are capable of giving rise to intact plants. This can be accomplished either by the promotion of stem tip growth and stimulation of axillary branching, or by inducing adventitious shoot buds either directly or through prior callus formation (Torres, 1989; George, 1993).

The structure is separated and subcultured onto new medium every 4-8 weeks. An important aspect of this stage is the maintenance of a high ratio of cytokinins to auxins which promotes shoot growth (Wetherall, 1982). However, nutrients, plant growth regulators and environmental factors all strongly influence shoot multiplication, and extensive testing is necessary to identify optimum conditions for efficient multiplication (George, 1993). Individual plant species and clones require different combinations and concentrations of nutrients, carbohydrates and plant growth regulators in order to be successfully propagated *in vitro* (Gamborg and Shyluk, 1981) and the type of multiplication medium used depends on the species or cultivar (Torres, 1989; Wetherall, 1989; George, 1993). The basal medium used during this procedure is similar to that used in the establishment phase; however, an increase in cytokinin and mineral supplement concentrations is supplied (Hartman *et al.*, 1997). Different species vary in their requirements for cytokinin and basal salts. Exact protocols for individual species thus have to be empirically determined through experimentation (Table 1.2).

The numbers of micro-shoots produced differs for individual explants, depending on the species and conditions under which cultures are maintained. Multiplication can be repeated several times by subculturing the explant onto fresh medium (George, 1993; Hartman *et al.*, 1997). This allows for an increase in the supply of material to a predetermined level for rooting and transplanting and any other *in vitro* manipulation. However, micro-shoots often deteriorate over time, lose leaves, fail to grow or lose their potential to regenerate, therefore it is appropriate to culture micro-shoots onto multiplication medium for a maximum of three subcultures (Hartman *et al.*, 1997).

The final stage of culture involves the formation of root and shoot systems that are strong enough to withstand the transition from *in vitro* conditions to those of independent growth in soil (Wetherell, 1982; Torres, 1989; George, 1993; Hartman *et al.*, 1997). The development of root systems in *in vitro* produced mico-shoots is an important process for the transfer of plantlets produced under

tissue culture conditions to those of independent growth conditions. Rooting can take place under *in vitro* or *ex vitro* growth conditions (George, 1993; Hartman *et al.*, 1997). During *in vitro* rooting the individual micro-shoots are transferred on to growth medium in which there is a change in the growth regulator concentration (Torres, 1989; George, 1993; Hartman *et al.*, 1997), by reducing the concentration of cytokinin and increasing the concentration of auxin. The concentration of the basal salts is also cut to half that present in the multiplication medium.

Considerable effort and research is necessary to identify the essential requirements of individual plant species in order to achieve success of *in vitro* propagation. Many methods have been developed to propagate several indigenous plants successfully under *in vitro* conditions (Table 1.2)

**Table 1.2:** Micropropagation protocols established for some indigenous plants with horticultural and medicinal properties.

| Plant species | Explant | Reference |
|---|---|---|
| *Mondia whiteii* | Single node explants derived from *in vitro* cultured seedlings | McCartan and Crouch, 1998 |
| *Tulbaghia simmleri* *Tulbaghia violoceae* | Culture of bulb explants | Zschocke and van Staden, 2000 |
| *Hypoxis* spp. | Direct and indirect plantlet regeneration from corms | Appleton and van Staden, 1995 |
| *Mesembryanthemum crystallinum* | Somatic embryogenesis with hypocotyl explants | Cushman *et al.*, 2000 |
| *Lachenalia* | leaf explants | Niederwieser and Vceler, 1990 |
| *Bowiea volubilis* | Proliferation of axillary shoots, initiation of adventitious shoots, | Hannweg *et al.*, 1996 |
| *Hyacinthaceae* | Induction of somatic embryos | McCartan and van Staden, 1999 |
| *Babiana* spp. | Culture of root, hypocotyl and leaf explants from *in vitro* germinated seedlings. | McAlister *et al.*, 1998 |
| *Crinum variabile* | Twin-scale explants | Fennell, *et al.*, 2001 |
| *Scilla kraussii* | Primary bulb explants | McCartan and van Staden, |
| *Scilla dracomontana* | | 2002. |
| *Scilla natalensis* | | McCartan and van Staden, 1998. |
| *Aloe polyphylla* | Shoot explants of *in vitro* grown plants | Chukwujekwu *et al.*, 2002 |
| *Ensete venticosum* | Zygotic embryos of stored seeds | Diro and van Staden, 2003 |
| *Harpagophytum procumbens* | Single node segments and shoot tips | Shushu, 2001 |
| *Podocarpus henkelil* | Apical regions of seedlings | Kowalski and van Staden, |
| *Podocarpus elongatus* | | 2001 |
| *Cussonia paniculata* | *In vitro* germinated seedlings | Tetyana and van Staden, 2001 |

*1.2.2.4 Practical constraints of plant tissue culture systems*

While it is possible to manipulate plant cells in tissue culture systems, the success of *in vitro* genetic manipulation will be limited if the practical constraints associated with plant tissue culture systems are not minimized. Several problems are encountered with *in vitro* plant tissue culture (Murashige, 1977), these include: hyperhydricity also known as vitrification (Rugini *et al.*, 1987; Kevers *et al.*, 2004), exudation of phenolics or internal browning (George, 1993; Hartman *et al.*, 1997), the appearance of contamination due to the presence of internal pathogens (Kunneman and Faaij-Groenen, 1988; Reed and Tanprasert, 1995) and shoot tip necrosis (Hartman *et al.*, 1997).

Hyperhydricity is characterized by the turgid appearance of *in vitro* shoots, which appear translucent and watery at their surface; hyperhydricity eventually results in cultures that deteriorate and fail to proliferate (DeBergh *et al.*, 1992; Hartman *et al.*, 1997). Poor rooting is encountered from hyperhydric shoots as they also do not survive the acclimatization step (Kevers *et al.*, 2004). Hyperhydricity is considered a physiological response resulting from the simultaneous stress factors of the *in vitro* culture medium, in addition to the high relative humidity in the culture vessel, and the accumulation of specific gases in the confined space (Kevers *et al.*, 2004). Jones *et al.* (1993) showed that hyperhydric *in vitro* cultured shoots of *Eucalyptus saligina* had abnormal, often discontinuous development of the epidermis and the cuticle. They also observed leaves that appeared thicker, and were characterized by poor differentiation between the palisade cells and spongy mesophyll. When the chlorophyll content and gross photosynthetic rate were determined, both were significantly lower than that of field-grown and normal cultured material (Jones *et al.*, 1993). Hyperhydricity is more frequently encountered in plants grown in liquid media or media with a low agar concentration, high humidity and high ammonium concentrations (Hartman *et al.*, 1997). A solution to this problem includes the addition of antihyperhydric agents to the culture medium (Hartman *et al.*, 1997). For example, McCartan and

Crouch (1998) showed that hyperhydricity was avoided in *M. whiteii* explants that were cultured on medium containing 0.2% activated charcoal while hyperhydricity was observed in shoots cultured on medium that lacked activated charcoal.

*In vitro* contamination by filamentous fungi, bacteria or yeasts is a critical problem in commercial and research laboratories (Kunneman and Faaij-Groenen, 1988; Reed and Tanprasert, 1995; Leifert and Cassells, 2001; Niedz and Bausher, 2002; Thomas, 2004). The source of contaminated cultures is difficult to identify, however this can stem from explants, laboratory environments, operators, mites and thrips, or ineffective sterilization techniques (Kunneman and Faaij-Groenen, 1988; Reed and Tanprasert, 1995; Leifert and Cassells, 2001). Contamination observed during the early stages of culture is often related to the bacterial and fungal status of the source plant (Kunneman and Faaij-Groenen, 1988). It is critical that every procedure of the plant tissue culture process is considered in order to prevent contamination. These procedures include handling of the stock plants, type and handling of the explants, media preparation, subculturing, incubation and storage of sterile cultures (Torres, 1989; George, 1993). Contamination that is detected during subculture can be due to inoculum that has gone unnoticed during the initial culture of the explant (Kunneman and Faaij-Groenen, 1988) or as a result of infection during transfer, originating from other contaminated cultures or the inefficient application of sterile techniques (Kunneman and Faaij-Groenen, 1988; Torres, 1989). The control of contamination involves the detection at an early stage and prevention of its spread through the cultures. Eliminating internal contaminants is difficult (Reed and Tanprasert, 1995; Leifert and Cassells, 2001). In some instances contamination can not be detected early because some contaminants mimic plant activities and therefore cannot be recognized as microbial in origin, for examples findings reported by Leifert and Waites (1990).

Explants of some species often turn black or brown within a few days after culture initiation (Torres, 1989; George, 1993; Hartman *et al.*, 1997). This results

in the inhibition of growth and usually death of the tissue (Torres, 1989). Browning is found to occur most frequently in those plant species that contain high levels of tannins or other hydroexophenols (Torres, 1989). Older tissues are generally more susceptible to browning than younger (Hartman *et al.*, 1997). The necrosis that does occur is a result of the action of copper-containing oxidase enzymes which are released due to the wounding that takes place during excision and sterilization of the explant (George, 1993; Hartman *et al.*, 1997).

There are several methods that can be employed to prevent browning of tissue, including removing the phenolic compounds produced (George, 1993; Hartman *et al.*, 1997), modifying the redox potential, inactivating the phenolase enzyme or reducing the phenolase activity (Torres, 1989). The most practical method of reducing browning has been the removal of phenolic substances (Torres, 1989).

There are several procedures that can be implemented to remove phenolic substances from a plant tissue culture system. The explant can be transferred frequently to fresh medium during the first 2-4 weeks of culture in order to reduce the build up of phenolic compounds, the frequency of transfer depending on the quantity of phenolic compounds synthesized by the species (Hartman *et al.*, 1997). The transfer of tissues can be facilitated by culturing them on liquid instead of solid medium, allowing the liquid medium containing the phenolics to be decanted and fresh medium to be added to the explant: this allows for minimum disturbance of the explant (Torres, 1989). When phenolics are bound to substances such as activated charcoal, they do not inhibit plant tissue culture growth. Concentrations of 0.5-0.05g.$l^{-1}$ of activated charcoal are generally added to the culture medium (Hartman *et al.*, 1997). Tissue browning can also be reduced by adjusting the redox potential with reducing agents or antioxidants (Torres, 1989). Tissues that are prone to browning can be dipped into a sterile solution of antioxidants such as ascorbic acid or citric acid immediately after excision (Torres, 1989).

Although various procedures can be implemented to ensure survival and successful regeneration of plant material *in vitro*, for those that have been subjected to induced mutations (such as *in vitro* polyploidization), screening procedures have to be implemented in order to establish if the mutation has been successfully induced. A number of screening procedures have been established to determine if polyploidization techniques have been successful in changing the ploidy status of a plant.

### 1.2.3 Ploidy analysis

Following *in vitro* polyploidization treatments with antimitotic agents it is necessary to determine if the treatment was successful. Success of the treatment depends on the frequency of induced polyploid plants that are produced and the rate of survival following treatment. Various techniques have been adopted to determine ploidy status. Macroscopic or microscopic screening for diagnostic features were used in early polyploidization investigations (Barret, 1974). Microscopical techniques have also been employed to count the number of chromosomes in treated cells (Gao *et al.*, 1996).

Cohen and Yao (1996) showed that rapid and non-destructive screening of populations of polyploids can be obtained using stomatal length measurements, and chromosomal counts can also be carried out on root tips. An advantage of using stomatal size to determine ploidy level is that it can be applied to emerging leaves, and this has been shown especially important for plants that are growing in the field (North, 1976). However, according to Awoleya *et al.* (1996) these techniques of identification are too laborious and ambiguous and may lead to mis-identification of mixaploid plants as solid polyploids, with subsequent instability during vegetative reproduction. Reliable estimates of nuclear DNA contents of different plant organs can be performed on isolated nuclei by using flow cytometry (Awoleya *et al.*, 1996). Only small amounts of tissue are necessary for analysis (Dolezel, 1997), and chromosomal stability in polyploid

material can be identified at very early stages from *in vitro* plantlets with minimal destructive harvesting (Awoleya *et al.*, 1996 and Dolezel, 1997). Several investigators have reported the efficiency of flow cytometry to evaluate changes in ploidy level after *in vitro* polyploidization treatment (Dolezel, 1997; Shao *et al.*, 2003 and Eeckhaunt *et al.*, 2004).

Flow cytometry is a technique that involves the analysis of the optical properties of particles in flow (Dolezel, 1997). Flow cytometry was originally developed for rapid counting and analysis of blood cells; however, with the development of new fluorescent probes, it is now a useful tool in many biological areas. Flow cytometry analysis to determine ploidy level in plants is usually carried out using nuclei isolated from young leaf tissue (Dolezel, 1997). Analysis of the relative DNA content yields a histogram showing a dominant peak corresponding to nuclei at the $G_1$ phase of the cell cycle. In order to estimate ploidy level, the position of the $G_1$ peak is compared with that of a reference plant of known ploidy. Flow cytometry assays used to determine ploidy status offer several important advantages over conventional chromosome counting (Dolezel, 1997). Sample preparation is easy, and therefore it is a convenient method; rapid analysis is possible therefore allowing several samples to be analysed over a shorter period of time; and there is no particular need to isolate meristematic tissue for analysis as flow cytometry does not depend on dividing cells. Samples can be prepared from a few milligrams of plant tissue, flow cytometry can be used to detect mixaploidy (Awoleya *et al.*, 1996).

### 1.2.4 Induced polyploidy in indigenous plant species

A consirable number of indigenous species have been polyploidized (Table 1.3). To achieve this, however, the inherent problems must be overcome as described by e.g. Niederwieser (2004) for *Lachenalia* spp.. One of the major constraints for successful commercialization of *Lachenalia*, an indigenous flower bulb species, has been slow propagation rates (Niederwieser, 2004) and several procedures have been implemented to overcome this, and technical advances have allowed for *in vitro* polyploidization to be applied on a routine basis to shorten the time required to develop new cultivars and to maintain the propagation scheme (Niederwieser, 2004). The techniques reported by that author include several reviews of *in vitro* polyploidization procedures carried out previously on *Lachenalia* spp.

At the Agricultural Research Council – Institute for Tropical and Subtropical Research (ARC-ITSR) in Nelspruit, many indigenous plant species have been polyploidized (Table 1.3) Hannweg and Vos (pers. comm. *). Explants used were nodal cuttings of *in vitro* germinated seedlings that were immersed for 24-48 hrs in liquid MS medium containing 0.01%, 0.05%, 0.1%, 0.5%, 1% or 5% colchcicine (w:v). The explants were recultured on MS medium containing 3% sucrose (w:v) and 0.3% Gelrite (w:v), and were maintained under reduced ventilation and high humidity conditions by sealing the culture tubes with Parafilm. The treated explants were evaluated six weeks later for changes in ploidy level using a flow cytometer. Treated explants that were tested positive for an increased ploidy level were transferred to a multiplication medium (MS medium supplemented with $30g.l^{-1}$ sucrose and $1mg.l^{-1}$ benzylaminopurine). Those treated explants that showed no change in polyploidy were re-routed into the antimitotic treatment stage and subsequently tested.

* Hannweg, K.F. and Vos, J.E. ARC-ITSR Private bag X11208, Nelspruit 1300

**Table 1.3:** A list of indigenous plant species that have been polypolidized at the ARC-ITSR with different concentrations (%) of colchicine Hannweg and Vos (pers. comm.).

| | % Colchicine | | | | | |
|---|---|---|---|---|---|---|
| **Plant species** | **0.01** | **0.05** | **0.1** | **0.5** | **1.0** | **5.0** |
| *Vigna unguiculata* | | | √ | | | |
| *Solanum retroflexum* | | | √ | √ | √ | √ |
| *Thymus vulgaris* | | | √ | | | |
| *Salvia aurea* | | | √ | | | |
| *Carpobrotus deliciousus* | | | √ | | | |
| *Ornithogalum suaveolens* | | | √ | | | |
| *Bauhinia galpinii* | √ | | √ | | | |
| *Albuca clanwilliams gloriae* | √ | | √ | | | |
| *Plectranthus fruitcosus* | √ | | | | | |
| *Coccinia quinqueloba* | | | √ | | | |
| *Citrullus lanatus* | √ | √ | √ | | | |
| *Romulea hallii* | √ | | √ | | | |
| *Mondia whiteii* | √ | | | | | |
| *Crocosmia aurea* | √ | | √ | | | |
| *Cucumis zeyheri* | | | √ | | | |
| *Dierama medium* | √ | | √ | | | |
| *Anthericum transvaalense* | √ | | √ | | | |
| *Phygelius capensis* | | | √ | | | |
| *Crotalaria capensis* | √ | | √ | | | |
| *Tranacetum vulgare* | √ | | √ | | | |
| *Eleusine coracana* | | | √ | | | |
| *Tephrosia rhodesica* | √ | | √ | | | |
| *Ocimum basilicum* | √ | | √ | | | |
| *Drimiopsis maculate* | | | √ | | | |
| *Dierama reynoldsii* | √ | | √ | | | |
| *Fagopyrum esculentum* | | | √ | | | |
| *Tetradenia riparia* | √ | | √ | | | |
| *Physalis peruviana* | √ | | √ | | | |
| *Sisymbrium thellungii* | | | √ | | | |
| *Sutherlandia spp.* | √ | | √ | | | |
| *Geranium incanum* | | | √ | | | |
| *Geranium pulchrum* | | | √ | | | |
| *Ceratotheca triloba* | | | √ | | | |
| *Tephrosia candida* | | | √ | | | |
| *Indigofera hedyanthe* | | | √ | | | |
| *Harpagophytum procumbens* | √ | | √ | | | |
| *Plectrantus spicatus* | | | | | √ | |
| *Hermannia athaeoides* | | | | | √ | |
| *Psoralea pinnata* | √ | | | | √ | |
| *Sanseiveira hyacinthoides* | | | | | √ | |
| *Dierama pendulum* | √ | | | | √ | |
| *Tulbaghia simmerlii* | √ | | | | √ | |
| *Pavonia columella* | √ | | | | √ | |
| *Indigofera filifolia* | | | | | √ | |
| *Barleria repens* | | | | | √ | |
| *Tecomaria capensis* | √ | | | | √ | |
| *Plantago lanceolata* | | | | | √ | |
| *Rumex acetosa* | √ | | | | √ | |
| *Ruta graveolens* | | | | | √ | |
| *Echinacea purpurea* | √ | | | | √ | |
| *Dodonae angustifolia* | | | | | √ | |
| *Sutherlandia frutescens* | √ | | | | √ | |
| *Sutherlandia speciosa* | | | √ | | | |

## 1.3 AIMS OF THE CURRENT INVESTIGATION

Many South African plant species have the potential for commercialization. However, a reduced distribution of naturally occurring resources has limited the isolation and cultivation of superior genotypes of several such potentially useful species, Hannweg and Vos (pers. comm.). In order to initiate successful cultivation of indigenous plant species, it is desirable that crop improvement strategies be employed. A convenient method that has been particularly useful with a spectrum of horticultural plant species is polyploidization or chromosomal doubling resulting from colchicine application, as described above. Polyploidization resulting from colchicine application has been a useful tool for crop improvement, especially when used in conjunction with *in vitro* plant tissue culture (Table 1.1).

At the ARC-ITSC in Nelspruit, successful *in vitro* chromosome doubling has been achieved with a number of indigenous plant species (Table 1.3). While successful polyploids have been produced in a number of indigenous plant species, only a limited number of genotypes of some species have been identified as polyploids and low survival rate of treated plant material have been common problems encountered when treating potential horticultural plants with colchcine, Hannweg and Vos (pers. comm.). The high concentrations of the antimitotic substance, colchicine, generally used to induce polyploidy are phytotoxic and carcinogenic. Another antimitotic substance that has been identified to induce polyploidy in plants is oryzalin. Oryzalin has been reported to induce similar responses in plants as that of colchicine but at a much lower concentration. The structures of colchicine and oryzalin are shown in Figure 1.1.

Although there are a number of reports highlighting the advantage of using oryzalin in place of colchicine to induce polyploidy *in vitro*, there is still uncertainty of the exact effects of the two antimitotic substances on cell ultrastructure. Therefore a comparison was made of the cell ultrastructure of plants that were

treated with colchicine and oryzalin in the present study. The present study focused on the efficiency of the two antimitotic substances in inducing poyploidy in two indigenous plant species with medicinal and horticultural properties (*Mondia whiteii* and *Dorotheathus bellidiformis*).

While *in vitro* polyploidization is considered the best method of inducing polyploidy, various practical constraints associated with plant tissue culture systems (see above) limit the survival of plant material that is treated with antimitotic substances, and hence limit the success of *in vitro* polyploidization treatments. To achieve maximum survival of explants treated with antimitotic substances it is critical that problems such as contamination, phenolics exudation, hyperhydricity and necrosis are reduced. It was therefore necessary in the present study to optimise the growth conditions of the treated plant material of the two species, prior to establishing which antimitotic substance was most efficient to induce polyploidy.

# CHAPTER TWO
# MATERIALS AND METHODS

## 2.1 EXPERIMENTAL APPROACH

To determine the effect of antimitotic substances on cellular structure, *Dorotheanthus bellidiformis* (Burm.f.) N.E.Br seeds were germinated *in vitro* in the presence of colchicine and oryzalin, and changes in ultrastructure and morphology were thereafter evaluated by light and electron microscopy. To determine the polyploidization efficiency of antimitotic substances, micro-shoots of *Mondia whiteii* (Hook.f.) Skeels and *D. bellidiformis* were treated with different concentrations of the antimitotic substances *in vitro* and then cultured under several different culture conditions, after which flow cytometry was used to evaluate the extent of polyploidization.

## 2.2 MATERIALS

### 2.2.1 Plant material

All plant material initially used was derived from seeds. *D. bellidiformis* (Figure 2.1a) seeds were purchased from McDonald Seeds, Pietermaritzburg and seeds of *M. whiteii* (Figure 2.1b) were purchased from Silverhill Seeds, Cape Town. The seeds were stored over silica gel in glass jars at room temperature and were sterilized prior to *in vitro* manipulation.

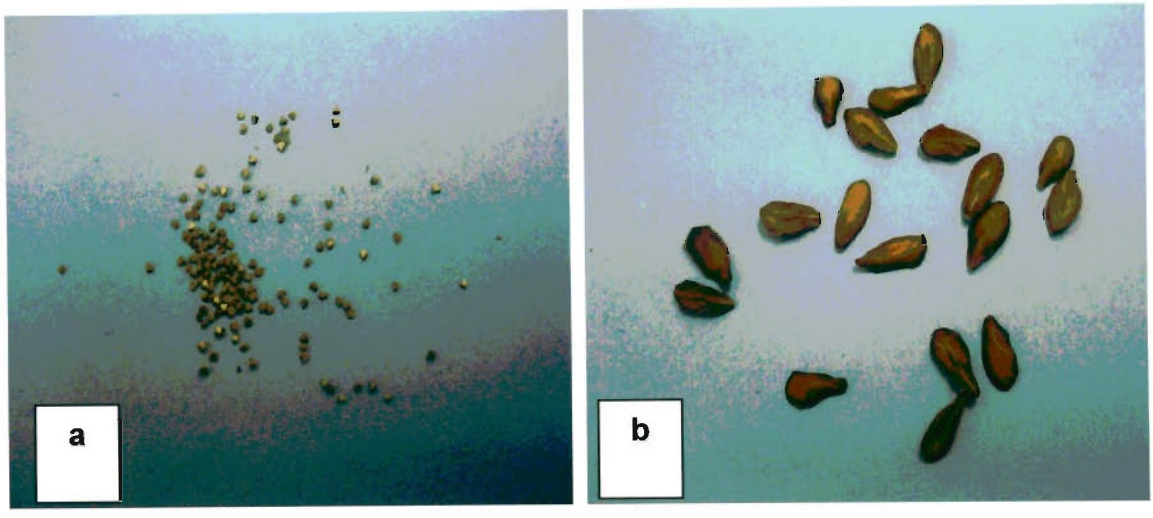


**Figure 2.1:** Seeds of (a) *D. bellidiformis* and (b) *M. whiteii* (x0.6).

## 2.3 EXPERIMENTAL METHODS

### 2.3.1 Sterilization

Seeds were initially immersed in 70% ethanol (v:v) for ±2 minutes. Floating debri and other particles were removed. The seeds were surface sterilized in a 1% solution of sodium hypochlorite (NaOCl) (w:v) containing a drop of Tween 20 for 0, 2, 4, 6 and 8 min, prior to thorough rinsing in three successive washes of sterile distilled water. The seeds were thereafter transferred aseptically onto Murashige and Skoog (1962) (MS) medium supplemented with 3% sucrose (w:v), and solidified with 0.3% Gelrite (w:v). The pH of the medium was adjusted to 5.7±0.02 prior to autoclaving.

The cultures were incubated at a 16 h/8 h photoperiod of 24°C day/ 21°C night for 14 days. Light was provided at a 37 $\mu mol.m^{-2}.s^{-1}$ Photosynthetic Photon Flux Density (PPFD) provided by Biolux tubes (Osram L58W) (lateral and overhead lighting). The number of seeds that germinated, the number of seeds showing contamination and those that failed to germinate was recorded. Four replicates of each treatment were carried out and ten seeds were used for each replicate.

The sterilization conditions at which optimal germination occurred was used in future investigations.

Statistical analysis was carried out using the statistical programme SPSS, the data was subjected to a one-way ANOVA. When the ANOVA indicated statistical significance, a Duncan's multiple comparison test was used to distinguish differences amongst means between treatments.

### 2.3.2 Germination of *D. bellidiformis* in the presence of antimitotic substances

Seeds were sterilized and placed on sterile filter paper moistened with autoclaved distilled water, 0.1% or 0.01% colchicine (w:v) and 0.01% and 0.001% oryzalin (w:v). The Petri dishes (θ 90.00 mm) were sealed with Parafilm and cultured for 14 days at a 16 h/8 h photoperiod of 24°C day / 21°C night and 37 $\mu mol.m^{-2}.s^{-1}$ PPFD.

Germination was recorded as initial radicle emergence over 14 days at which point the germinated seedlings were transferred to MS medium. Ten replicates of each treatment were carried out and 20 seeds were used for each replicate. 0.05 $g.ml^{-1}$ and 0.01 $g.ml^{-1}$ stock solutions of colchicine and oryzalin respectively were made up using sterile distilled water and a few drops of dimethyl sulphoxide (DMSO) (Sanders and Hull, 1970). The stock solutions of the antimitotic substances were added to distilled water prior to autoclaving.

Statistical analysis was conducted using the statistical programme SPSS and the data was subjected to a one-way ANOVA. When the ANOVA indicated statistical significance, a Duncan's multiple comparison test was used to distinguish differences amongst means between treatments.

### 2.3.3 Post-treatment handling of seedlings

After 14 days in the presence of antimitotic substances, germinated seedlings were transferred to MS medium containing 3% sucrose (w:v), 0.3% Gelrite (w:v) at pH 5.7±0.02 in Petri dishes (θ 90.00 mm) for 2 weeks at a 16 h / 8 h photoperiod of 27°C day/ 21°C night and PPFD of 66 $\mu mol.m^{-2}.s^{-1}$. The pattern of growth of treated seedlings was recorded using a Sony F.D. Movica digital camera.

Apical and root meristems were excised from treated seedlings 14 days after treatment. The excised tissue was fixed in 2.5% glutaraldehyde buffered at pH 7.2 with 0.1 M phosphate buffer containing 1% caffeine for at least 24 h and post fixed in 0.5% aqueous osmium tetroxide for an hour. The material was dehydrated with 25%, 50%, 75% and 100% acetone (v:v). The dehydrated samples were placed in 1:1 100% acetone and resin (Spurr, 1969) for 4 h, placed in resin (Spurr, 1969) for 24 h and thereafter embedded in fresh resin and polymerised at 80°C for 8 h. Five replicates of each sample were prepared for microscopy.

The embedded plant material was sectioned using an LKB ultramicrotome. Sections viewed for light microscopy were 1 μm thick and stained with 1% toluidine blue. Sections viewed for transmission electron microscopy cut at 100±0.15 nm, and double stained with aqueous 2.5% uranyl acetate solution for 10 min followed by lead citrate (Reynolds, 1963) for 10 min. A maximum of 5 sections were viewed on each grid. The sections were viewed and photographed on Kodak film using a Jeol 1010 transmission electron microscope.

A qualitative analysis was carried out at a descriptive level of the micrographs obtained from TEM, and particular attention was paid to organelle development, vacuolation and any possible abnormalities indicative of cell damage.

## 2.4 *IN VITRO* POLYPLOIDIZATION

### 2.4.1 Explant preparation

Sterilized *D. bellidiformis* and *M. whiteii* seeds were germinated on MS medium containing 3% sucrose (w:v) and 0.3% Gelrite (w:v) at pH 5.7±0.02. Seeds were germinated individually in culture tubes sealed with Parafilm containing 10 ml of medium. Seedlings were maintained for six weeks on the MS medium until they were 5±0.5 cm in length.

At least 3 nodal cuttings (1.5 cm) were made from the seedlings and transferred onto MS medium supplemented with 3% sucrose (w:v); 0.3% Gelrite (w:v) and 1 $mg.l^{-1}$ benzyl aminopurine (BAP) at a pH of 5.7±0.2 and maintained at a 16 h/ 8 h photoperiod of 24°C day / 21°C night at PPFD of 37 $\mu mol.m^{-2}.s^{-1}$. Three subcultures onto fresh multiplication medium after six week intervals were necessary to obtain sufficient number of micro-shoots of 0.5 – 1 cm lengths for the manipulations that followed.

1 $mg.ml^{-1}$ stock solution of BAP (Sigma, U.S.A) was used to prepare the multiplication medium. This was made by dissolving 1 g of BAP in 1 M NaOH and thereafter made up to a volume of 100 ml with sterile distilled water. The stock solution was stored in an amber glass bottle in the refrigerator, and was removed from the refrigerator at least an hour before being used to prepare media.

### 2.4.2 Antimitotic treatment of in vitro micro-shoots

*In vitro* produced micro-shoots (0.5 cm) were excised from individual clonal cultures of *D. bellidiformis* (Figure 2.2a) and *M. whitei* (Figure 2.2b) and immersed in 1.5 ml of liquid MS medium supplemented with 3% sucrose (w:v), at a pH of 7.00±0.02 (Figure 2.2c). The liquid medium was further supplemented with 0.1% or 0.01% colchicine (w:v) and 0.01% or 0.001%

oryzalin (w:v) and dispensed into 2 ml cryo-tubes. These cultures were maintained in the liquid medium for 48 h at a16 h/ 8 h photoperiod of 24°C day / 21°C night at PPFD of 37 $\mu mol.m^{-2}.s^{-1}$.

Explants were thereafter thoroughly rinsed individually with 3 successive washes of sterile distilled water, and transferred onto an elongation medium. Micro-shoots subjected to control treatments were treated in the same manner but were cultured in liquid medium without any antimitotic substances. Five experimental treatments were carried out using 20 replicates for each treatment.

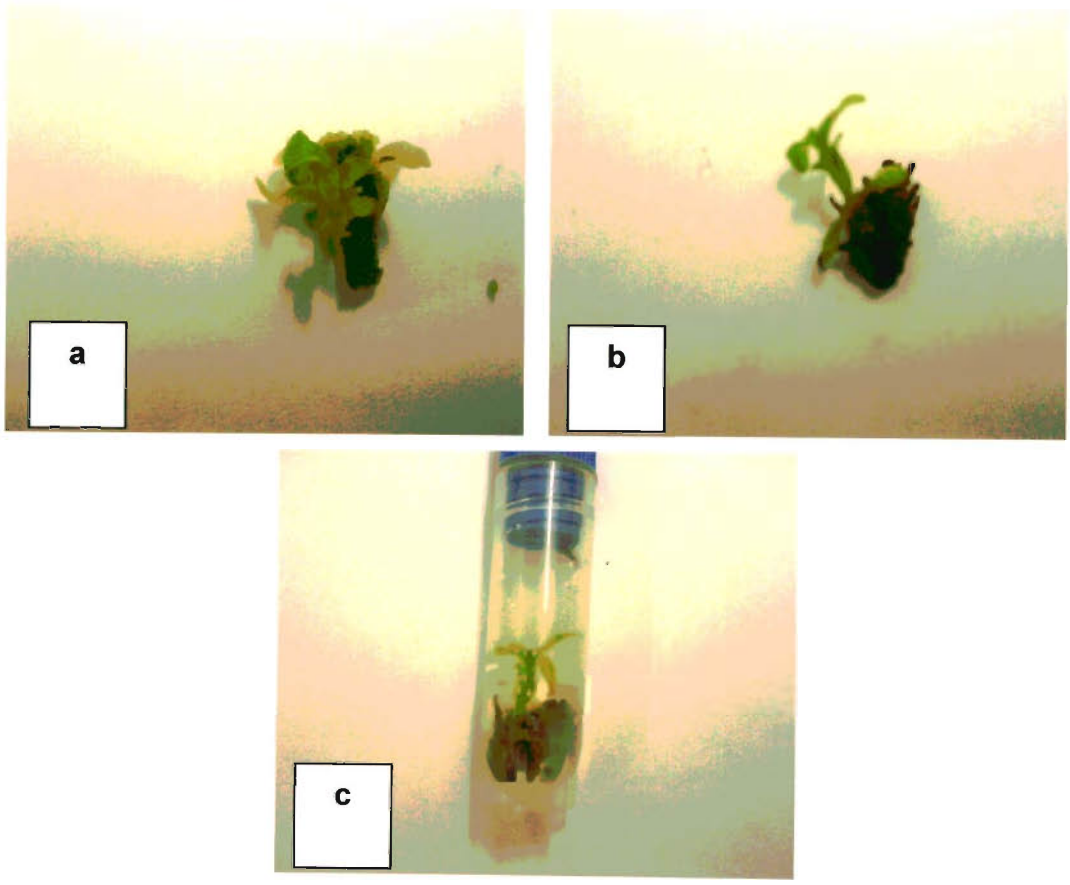


**Figure 2.2:** Clonal explants of (a) *D. bellidiformis* and (b) *M. whiteii* treated with antimitotic substances in liquid MS medium in (c) 2 ml cryotubes (ø 1 cm) (x0.75).

### 2.4.3 *In vitro* culture of treated micro-shoots

The rinsed micro-shoots were individually transferred to 5 ml of elongation medium in 20 ml culture tubes. Optimum micro-shoot elongation was assessed after the following procedures:

a. Initially micro-shoots of *D. bellidiformis* and *M. whiteii* explants were transferred onto MS medium supplemented with 3% sucrose (w:v), 0.3% Gelrite (w:v) at a pH of 5.7±0.02. Individual culture tubes were sealed with Parafilm and maintained for 6 weeks.

b. Micro-shoots were maintained for 6 weeks on 5 ml of MS medium supplemented with 3% sucrose (w:v), 0.3% Gelrite (w:v) at a pH of 5.7±0.02 but were cultured in tubes with caps but were not sealed with Parafilm.

c. Treated micro-shoots were cultured for four and six weeks on MS medium supplemented with 3% sucrose (w:v), 0.3% Gelrite (w:v), 0.2% activated charcoal (w:v) at pH 5.7±0.02.

d. All treated micro-shoots of *M. whiteii* were cultured at a 16 h / 8 h photoperiod of 24°C day/ 21°C night and PPFD of 37 $\mu mol.m^{-2}.s^{-1}$. Treated micro-shoots of *D. bellidiformis* were cultured at 16 h / 8 h photoperiod of 27°C day/ 21°C night and PPFD of 66 $\mu mol.m^{-2}.s^{-1}$.

Treated micro-shoots were evaluated at the end of the culture period for hyperhydricity, phenolics exudation, survival, contamination and overall growth response to the treatments. Elongated micro-shoots produced under conditions where optimum growth was observed, were evaluated for changes in ploidy level.

## 2.5 PLOIDY ANALYSIS

Leaf samples (Figure 2.3a) were excised with a sharp razor blade from treated micro-shoots after culture on an appropriate elongation medium. The excised samples were placed in 2 ml of sterile distilled water in Eppendorf tubes (Figure 2.3b), sealed with Parafilm, and transferred to the Agricultural Research Council- Institute for Tropical and Subtropical Crop at Nelspruit for ploidy analysis.

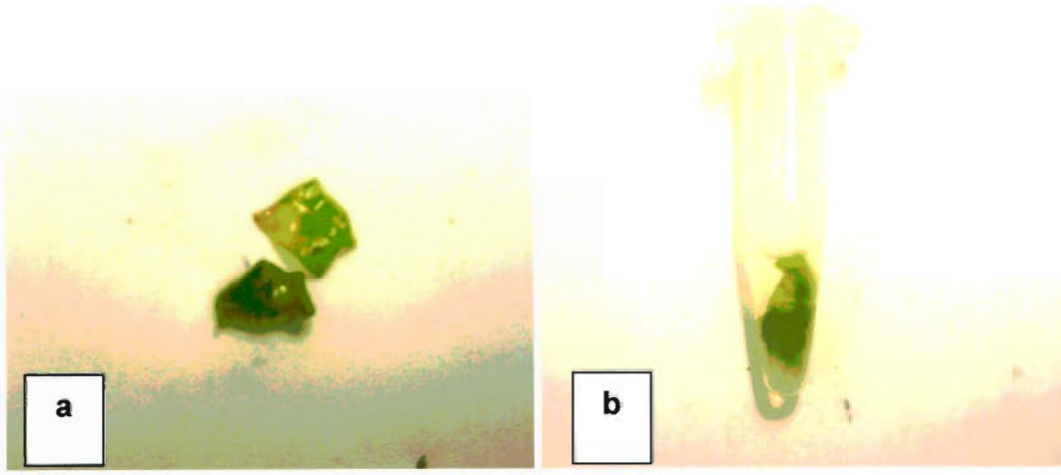


**Figure 2.3:** Leaf samples from treated micro-shoots (a) were transferred for flow cytometry analysis in Eppendorf tubes (b) containing 2 ml of sterile distilled water (x0.85).

Flow cytometry was used to determine the changes in ploidy level of treated micro-shoots using a Partec, PA Ploidy Analyser. Leaves were first chopped with a sharp razor blade in a 55 mm plastic Petri dish containing 400 µl of nucleus extraction buffer. The sample was then filtered through a 50 µm Celltrics disposable filter into plastic cuvettes and 1.6 ml of 4',6'-diamidino-2-phenylindol (DAPI) staining solution was added. The change in ploidy level was measured with a flow cytometer. The principles of this method have been presented above (p 34).

# CHAPTER THREE
# RESULTS

## 3.1 RESPONSE OF SEEDS TO STERILIZATION TREATMENTS

An important aspect of *in vitro* manipulation of plant material is the removal of contaminants. In the current study, explants (seeds) were surface sterilized with a commonly used sterilizing agent (NaOCl) for various lengths of time (0, 2, 4, 6 and 8 min) and screened for a period of 2 weeks on a solid sucrose-supplemented MS medium for the presence of contaminants and germination. Once an optimum period for sterilization was determined for *D. bellidiformis*, the same sterilization conditions were used for *M. whiteii* seeds.

Immersion of *D. bellidiformis* seeds in 1% NaOCl (w:v) for different lengths of time showed differences in the germination response when the seeds were subsequently cultured *in vitro* on MS medium (Table 3.1). Low germination (10%) was recorded for seeds that were cultured directly onto sucrose-supplemented MS medium after only a 2 min immersion in 70% ethanol, and contamination was also high (87%). The germination of the seeds increased as the duration of immersion in 1% NaOCl was increased. A relatively high germination (75%) was recorded for seeds immersed in 1% NaOCl (w:v) for 8min.

An inverse relationship was observed for seeds lost to contamination with increasing the length of time immersed in 1% NaOCl. There were no significant differences observed for seeds that failed to germinate when immersed in 1% NaOCl for 0 – 8 min. The mean percentage of seeds failing to germinate was relatively low in comparison with seeds that germinated under all treatment regimes. Seeds lost to contamination, and seeds failing to germinate together, were lowest for seeds treated with 1% NaOCl for 8 min in comparison with treating seeds with 1% NaOCl for shorter periods. Contamination recorded for seeds immersed for 8 min in 1% NaOCl was relatively low (15%). Increasing the sterilization period to 10 and 12 min

inhibited germination completely. *Mondia whiteii* seeds were immersed for 8 min in 1% NaOCl followed by 3 successive washes with sterile distilled water showed 100% germination. Nodal cuttings of uncontaminated seedlings were placed on multiplication medium (sucrose-supplemented MS medium containing 1mg.$l^{-1}$ BAP) in order to generate an adequate supply of *in vitro* micro-shoots for subsequent *in vitro* polyploidization studies.

An initial 2 min immersion in 70% ethanol followed by 8 min in 1% NaOCl and thereafter followed by 3 successive washes with sterile distilled water was used for future sterilization of both *D. bellidiformis* and *M. whiteii* seeds when *in vitro* manipulation of these two species was carried out.

**Table 3.1:** Sterilization and germination responses of *D. bellidiformis* seeds to 1% sodium hypochlorite for varying periods of time after 14 days. The sterilized seeds were cultured on full strength MS medium supplemented with 3% sucrose (w:v) and 0.3% Gelrite at a pH of 5.7±0.02 and a 16 h day /8 h night photoperiod of 24°C day / 21°C night and PPFD of 37 µmol.$m^{-2}$.$s^{-1}$.

| Treatment duration (min) | Germination (%) | Contamination (%) | No germination (%) |
|---|---|---|---|
| 0 | 10±5.8$^{a}$ | 88±7.5$^{a}$ | 3±2.5$^{a}$ |
| 2 | 25±10.4$^{ab}$ | 73±11.1$^{ab}$ | 3±2.5$^{a}$ |
| 4 | 55±19.4$^{bc}$ | 43±18.9$^{bc}$ | 3±2.5$^{a}$ |
| 6 | 63±11.1$^{bc}$ | 35±8.7$^{bc}$ | 3±2.5$^{a}$ |
| 8 | 75±15.6$^{c}$ | 15±11.9$^{c}$ | 8±4.8$^{a}$ |

$^{z}$Mean separation within columns by Duncans' multiple range test, $P \leq 0.05$. Values represent means and standard error of means of four replicates of 20 seeds each.

## 3.2 RESPONSES OF *D. BELLIDIFORMIS* SEEDS TO ANTIMITOTIC SUBSTANCES

### 3.2.1 Germination response

*Mondia whiteii* seeds were used exclusively to derive an adequate stock of *in vitro* micro-shoots for *in vitro* manipulation of chromosome number. *Dorotheanthus bellidiformis* seeds were used to derive stocks of *in vitro* micro-shoots for *in vitro* chromosome studies, as well as to determine the effect of the antimitotic substances (oyzalin and colchicine) on a highly metabolic process (germination) and evaluate the subsequent alteration of cell ultrastructure. Seeds of *D. bellidiformis* were first sterilized (2 min in 70% ethanol, 8 min in 1% NaOCl followed by three successive rinses in sterile distilled water) and germinated *in vitro* in the presence of 0.1% or 0.01% colchicine and 0.01% or 0.001% oryzalin. Percentage germination was recorded daily from day 3, at which time initial signs of germination were observed, with the protrusion of the radicle and swelling of the seed coat. A significant difference in germination rate was observed for seeds germinated in the presence of 0.01% oryzalin compared with controls (Table 3.2). There was no significant difference observed between seeds germinated at 0.1% and 0.01% colchicine, 0.001% oryzalin and control treatments (Table 3.2). The percentage germination increased steadily for *D. bellidiformis* cultured *in vitro* in the presence of 0.1% and 0.01% colchicine, 0.01% and 0.001% oryzalin over the 14 days observed (Table 3.2).

The initial germination recorded for seeds of the control treatments, 0.1% and 0.01% colchicine (w:v) and 0.001% oryzalin (w:v) was rapid by day three, but thereafter was followed by a slower progressive increase in percentage germination (Table 3.2). A low percentage germination (10%) was recorded at 0.01% oryzalin initially, which gradually increased to 44% by day 14.

The general trend for all treatment conditions appeared to be a rapid germination during the first three days followed by a slow, steady increase in germination thereafter. Culturing of seeds in the presence of 0.01% oryzalin

reduced the final germination rate observed by nearly half, suggesting that the treatment was phytotoxic.

Differences in morphology after 14 days (Figure 3.1) were observed on initial transfer of germinated seedlings to the sucrose-supplemented MS medium (Figure 3.1). No observable differences were observed in appearance of seedlings germinated under control conditions (Figure 3.1b) and those in the presence of 0.01% colchicine (image of 0.01% treated seedling not presented a it does not vary much in appearance from seedlings treated as controls). Seedlings germinated at the two colchicine concentrations were markedly different: root development in seedlings germinated at 0.01% colchicine was vigorous and shoot elongation was shown, which was similar to that observed in control seedlings (Figure 3.1b) whereas stunting was evident at 0.1% colchicine (Figure 3.1a).

Root development in seedlings germinated in the presence of colchicine (Figures 3.1a), and control germinated seedlings (Figure 3.1b) were different from rooting observed in seedlings germinated in the presence of oryzalin at both concentrations tested (Figure 3.1c-d). Oryzalin treated seedlings were markedly stunted in development and differed morphologically from control seedlings, and those germinated in the presence of colchicine. Seedlings germinated at 0.01% and 0.001% oryzalin (Figure 3.1c) lacked pigmentation, and appeared translucent. Restricted root development was observed in oryzalin treated seedlings (Figures 3.1c and d). Limited shoot development was observed in seedlings that were treated with 0.01% oryzalin (Figures 3.1c).

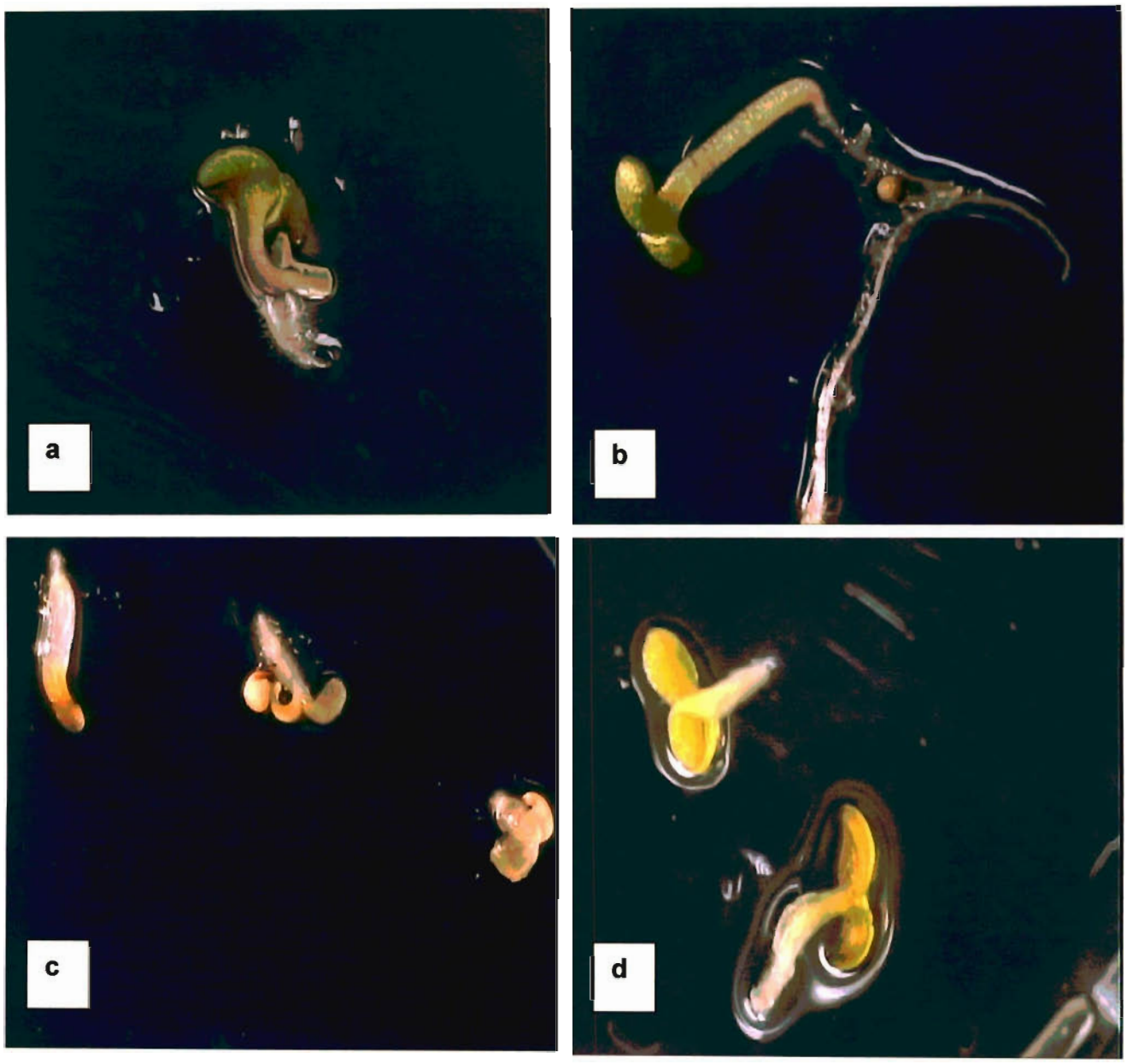


**Figure 3.1:** Morphological appearance of seedlings germinated in the presence of antimitotic substances for 14 days: a – 0.1% colchicine; b – control; c - 0.01% oryzalin and d – 0.001% oryzalin.

**Table 3.2:** *In vitro* percentage germination of *D. bellidiformis* seeds initially subjected to an 8 min sterilization regime and thereafter cultured on sterile filter paper moistened with: distilled water or 0.1% colchicine, 0.01% colchicine, 0.01% oryzalin or 0.001% oryzalin under a 16 h day / 8 h night photoperiod of 24°C day / 21°C night and PPFD of 37 $\mu mol.m^{-2}.s^{-1}$.

| Treatments | Germination (%) at day | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 |
| Control | 56±2$^{a}$ | 66±3$^{a}$ | 66±3$^{a}$ | 73±3$^{ab}$ | 75±3$^{a}$ | 78±3$^{a}$ | 78±3$^{a}$ | 78±3$^{ab}$ | 79±3$^{ab}$ | 79±3$^{ab}$ | 80±3$^{a}$ | 80±3$^{a}$ |
| 0.1% colchcine | 42±4$^{a}$ | 66±3$^{a}$ | 71±4$^{a}$ | 74±4$^{a}$ | 76±4$^{a}$ | 76±4$^{a}$ | 76±4$^{a}$ | 76±4$^{ab}$ | 77±4$^{ab}$ | 77±4$^{ab}$ | 77±4$^{a}$ | 78±4$^{a}$ |
| 0.01% colchicine | 48±6$^{ab}$ | 56±6$^{ab}$ | 58±6$^{ab}$ | 72±5$^{ab}$ | 76±5$^{ab}$ | 78±5$^{a}$ | 78±5$^{a}$ | 78±5$^{ab}$ | 81±5$^{a}$ | 80±5 | 82±4$^{a}$ | 82±4$^{a}$ |
| 0.01% oryzalin | 10±4$^{c}$ | 18±5$^{c}$ | 17±5$^{c}$ | 33±8$^{c}$ | 35±8$^{c}$ | 39±8$^{b}$ | 39±8$^{b}$ | 40±8$^{c}$ | 40±8$^{c}$ | 40±8$^{c}$ | 41±8$^{b}$ | 44±8$^{b}$ |
| 0.001% oryzalin | 33±5$^{b}$ | 42±7$^{b}$ | 42±7$^{b}$ | 66±8$^{ab}$ | 62±8$^{ab}$ | 63±8$^{a}$ | 63±8$^{a}$ | 63±8$^{b}$ | 62±8$^{b}$ | 62±8$^{b}$ | 66±8$^{a}$ | 67±7$^{a}$ |

[z]Mean separation within vertical columns by Duncans' multiple range test, $P \leq 0.05$. Values represent means and standard error of means of 10 replicates of 25 seeds each.

### 3.2.2 Morphological and ultrastructural responses

Although the initial germination responses for the controls, and seeds cultured in the presence of 0.1% and 0.01% colchicine and at 0.001% oryazalin initially appeared similar, differences (Figures 3.3-3.7) became evident after transferring the treated seedlings onto sucrose-supplemented MS medium.

Seedlings from control treatments (Figures 3.3a-c) showed vigorous root growth when transferred to the sucrose-supplemented MS medium. By day 6 of culture (Figure 3.3b), rapid elongation of micro-shoots was evident, and root development was extensive with numerous root hairs. At the termination of the culture period on day 14, a dense mass of plantlets, with extensively elongated shoots and vigorous primary roots and root hairs were observed (Figure 3.3c).

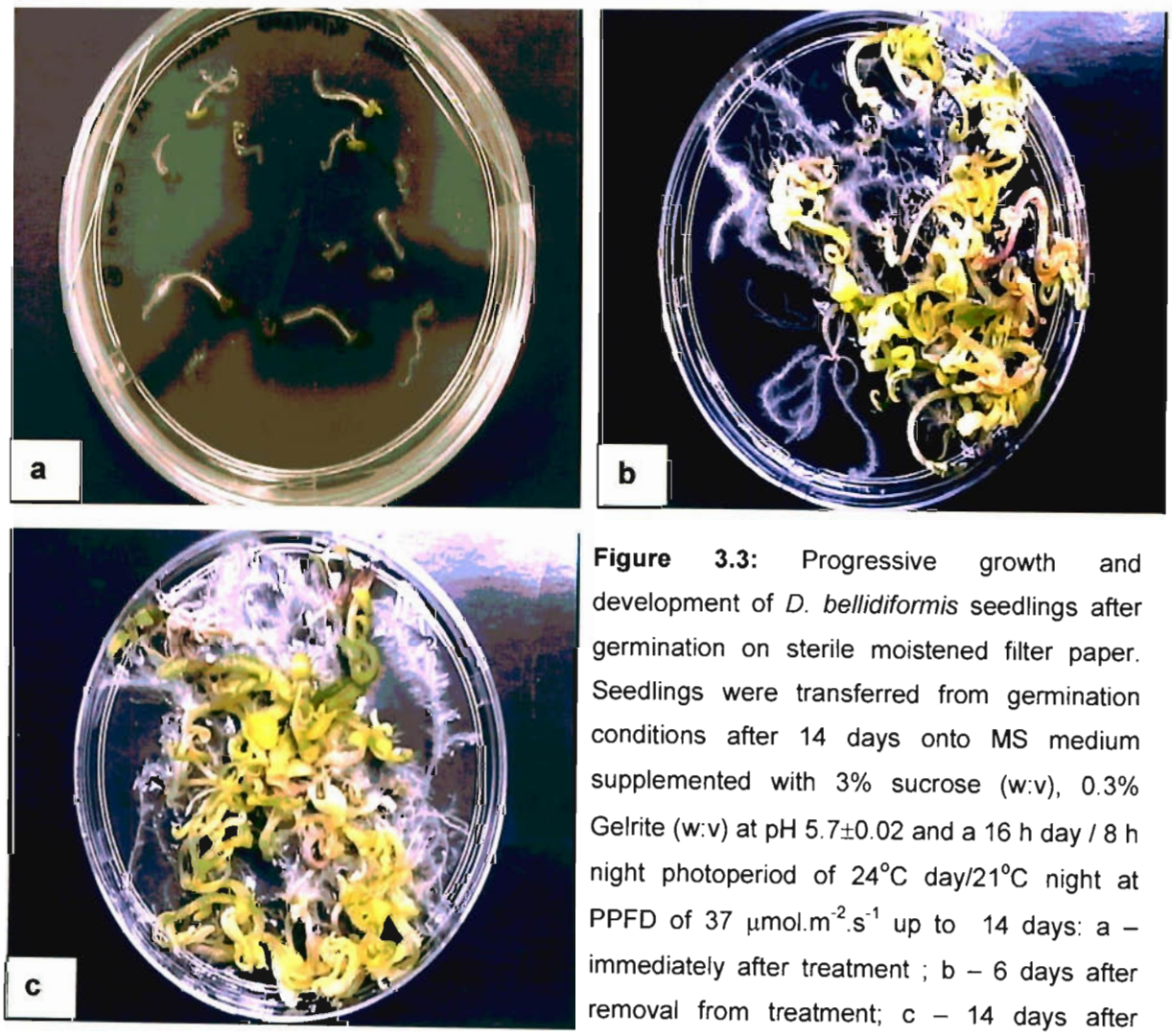


**Figure 3.3:** Progressive growth and development of *D. bellidiformis* seedlings after germination on sterile moistened filter paper. Seedlings were transferred from germination conditions after 14 days onto MS medium supplemented with 3% sucrose (w:v), 0.3% Gelrite (w:v) at pH 5.7±0.02 and a 16 h day / 8 h night photoperiod of 24°C day/21°C night at PPFD of 37 $\mu mol.m^{-2}.s^{-1}$ up to 14 days: a – immediately after treatment ; b – 6 days after removal from treatment; c – 14 days after

Plantlet growth was slow in seedlings treated with 0.1% colchicine (Figure 3.4) and although rooting was evident, it was far less extensive than the controls. Photosynthesis was presumably occurring in these seedlings, as their leaves were dark green. Cotyledonary greening appeared unaffected by the treatment and at the termination of culture on day 14, unlike control seedlings, shoot elongation was reduced and root development was not as vigorous (Figure 3.4c compared with Figure 3.3c).

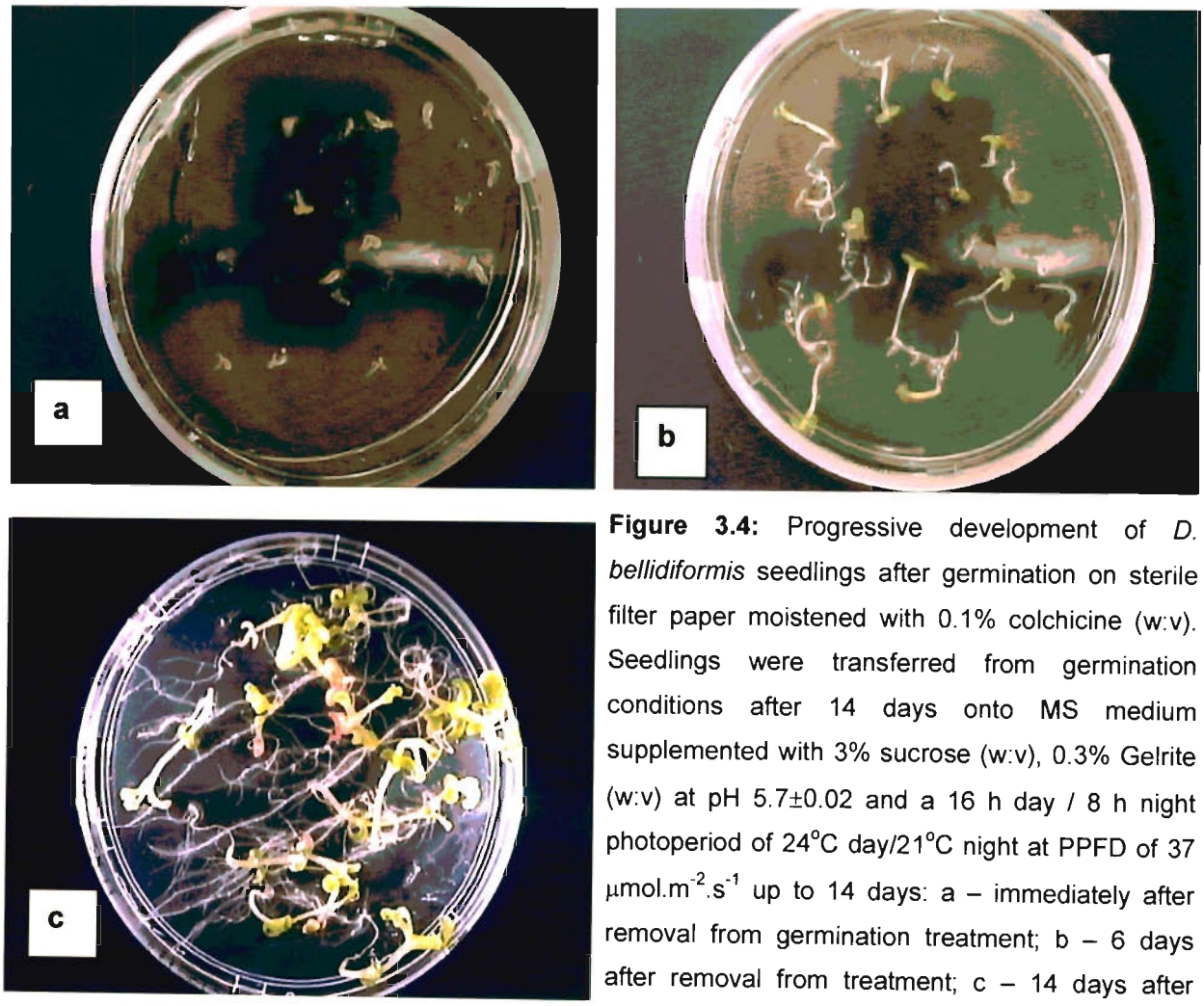


**Figure 3.4:** Progressive development of *D. bellidiformis* seedlings after germination on sterile filter paper moistened with 0.1% colchicine (w:v). Seedlings were transferred from germination conditions after 14 days onto MS medium supplemented with 3% sucrose (w:v), 0.3% Gelrite (w:v) at pH 5.7±0.02 and a 16 h day / 8 h night photoperiod of 24°C day/21°C night at PPFD of 37 $\mu mol.m^{-2}.s^{-1}$ up to 14 days: a – immediately after removal from germination treatment; b – 6 days after removal from treatment; c – 14 days after removal from treatment.

Although seedlings removed from 0.01% colchicine were initially similar to seedlings from control conditions, growth patterns observed for seedlings germinated at 0.01% colchicine changed as development proceeded (Figure 3.5). Inhibition of shoot development was still evident 6 days after transferring the seedlings to the MS medium (Figure 3.5b) compared with those of control treatments (Figure 3.3b), however, inhibition of shoot elongation was much less for seedlings germinated at 0.01% colchicine than that which was evident for seedlings of the same age germinated at 0.1% colchicine (Figure 3.4b). Hypocotyl growth was evident 14 days after transferring seedlings onto MS medium (Figure 3.5c), but cotyledonary development appeared stunted (Figure 3.5c) and this could be compared with cotyledonary development of control seedlings of the same age (Figure 3.3c).

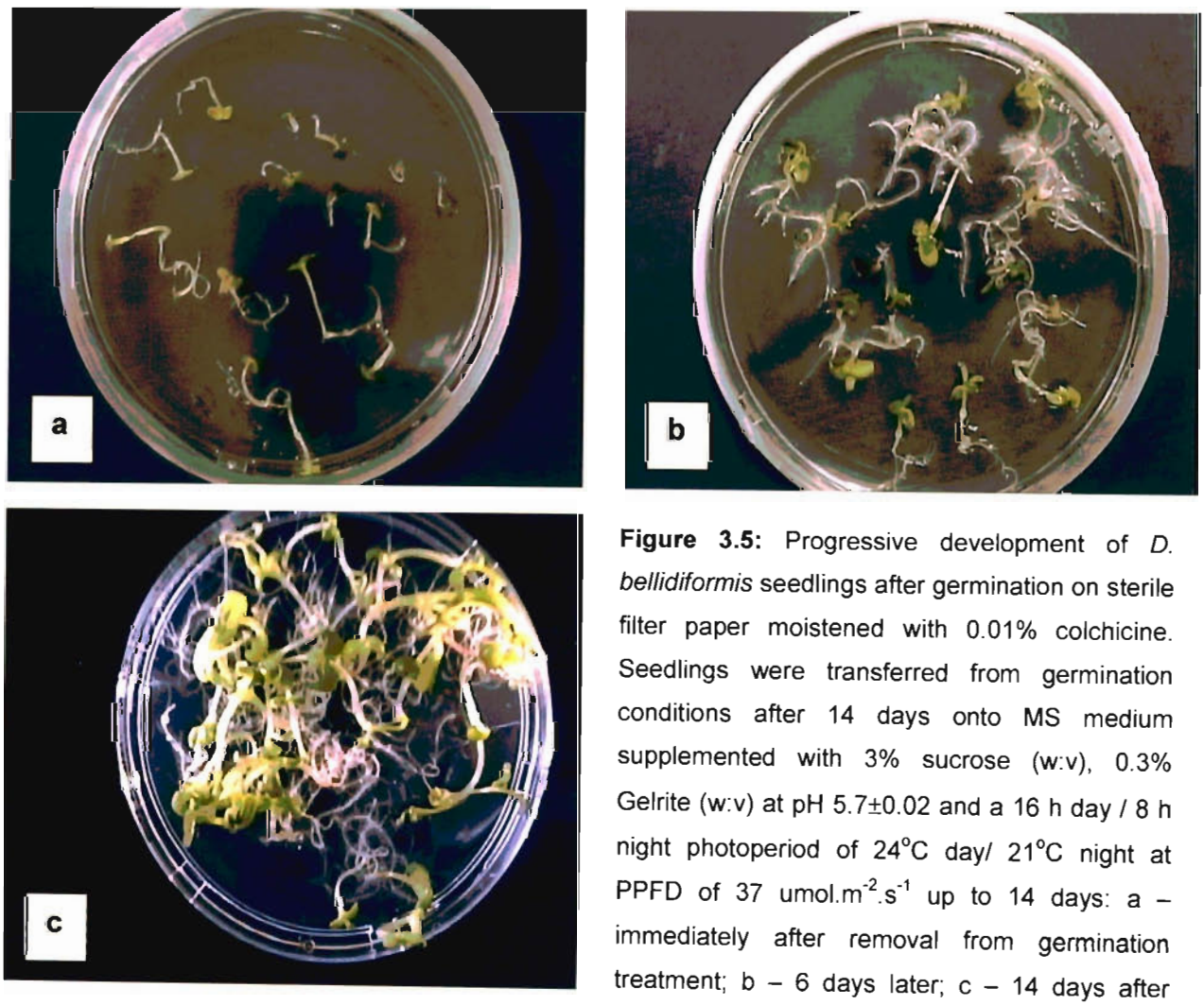


**Figure 3.5:** Progressive development of *D. bellidiformis* seedlings after germination on sterile filter paper moistened with 0.01% colchicine. Seedlings were transferred from germination conditions after 14 days onto MS medium supplemented with 3% sucrose (w:v), 0.3% Gelrite (w:v) at pH 5.7±0.02 and a 16 h day / 8 h night photoperiod of 24°C day/ 21°C night at PPFD of 37 $umol.m^{-2}.s^{-1}$ up to 14 days: a – immediately after removal from germination treatment; b – 6 days later; c – 14 days after removal from treatment.

Growth patterns observed in seedlings germinated in the presence of oryzalin (Figure 3.6 and Figure 3.7) were remarkably different from the growth patterns observed in controls (Figure 3.3) and those germinated in the presence of colchicine (Figure 3.4 and Figure 3.5). The inhibitory effect of 0.01% oryzalin on seedling growth and development (Figure 3.6) was striking in relation to colchicine treatments and water controls.

An inhibition in root development was observed when seedlings were initially transferred onto a sucrose-supplemented MS medium after a 14 day treatment with 0.01% oryzalin (Figure 3.6a) compared with controls (Figure 3.3a). While most seedlings treated with 0.01% oryzalin showed no shoot elongation after they were transferred onto MS medium (Figure 3.6c) compared with that in seedlings from control treatments (Figure 3.3b), retarded elongation was observed in the few seedlings that did show signs of development (Figure 3.6c). At the end of the culture period only a few seedlings developed further, and primary root development (Figure 3.6c) was still slow and no root hair development was observed compared with control seedlings (Figure 3.3c) at the same developmental stage. Only a limited number of seedlings treated with 0.01% oryzalin were green (Figure 3.6c) suggesting reduced photosynthetic activity.

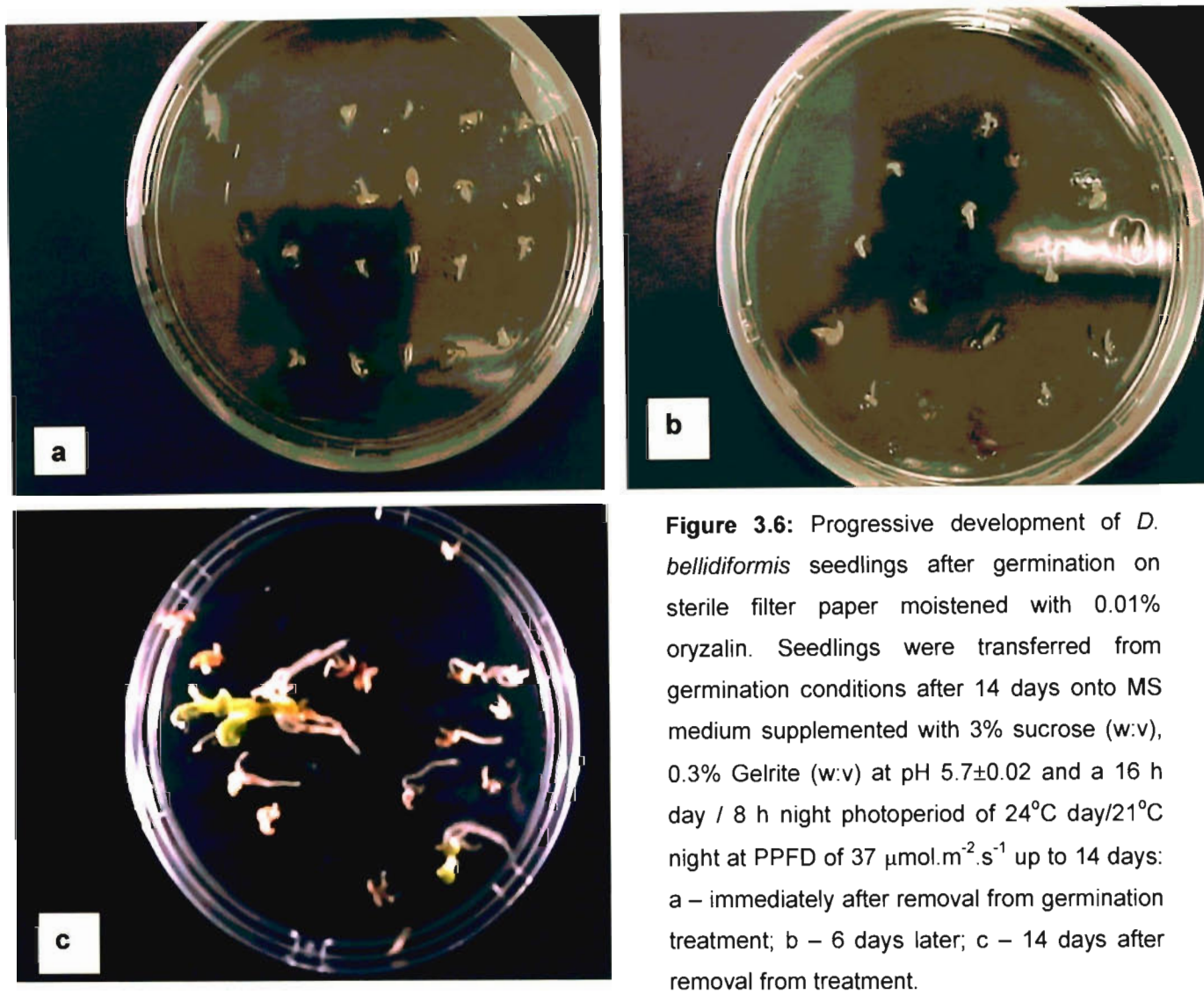


**Figure 3.6:** Progressive development of *D. bellidiformis* seedlings after germination on sterile filter paper moistened with 0.01% oryzalin. Seedlings were transferred from germination conditions after 14 days onto MS medium supplemented with 3% sucrose (w:v), 0.3% Gelrite (w:v) at pH 5.7±0.02 and a 16 h day / 8 h night photoperiod of 24°C day/21°C night at PPFD of 37 $\mu mol.m^{-2}.s^{-1}$ up to 14 days: a – immediately after removal from germination treatment; b – 6 days later; c – 14 days after removal from treatment.

Seedlings isolated after a treatment with 0.001% oryzalin (Figure 3.7) appeared less severely inhibited than those treated with 0.01% oryzalin (Figure 3.6). Growth of seedlings that were treated with 0.001% oryzalin and thereafter transferred onto a sucrose-supplemented MS medium were inhibited, but not as markedly as that observed for 0.01% oryzalin treated seedlings (Figure 3.6) Although root development was restricted following removal of the seedlings from the treatment, some hypocotyl elongation was evident (Figure 3.7a). Although shoot development was evident 6 days after transferring the treated seedlings to the sucrose-supplemented MS medium (Figure 3.7b), it was still

relatively slow when compared to control seedlings of the same age (Figure 3.3b). Thereafter, shoot growth proceeded slowly and steadily (Figure 3.7c). Root development was restricted (Figure 3.7c) compared with seedlings from the control treatments (Figure 3.3b), but was evident 14 days after transferring the seedlings onto the sucrose-supplemented MS medium (Figure 3.7c).

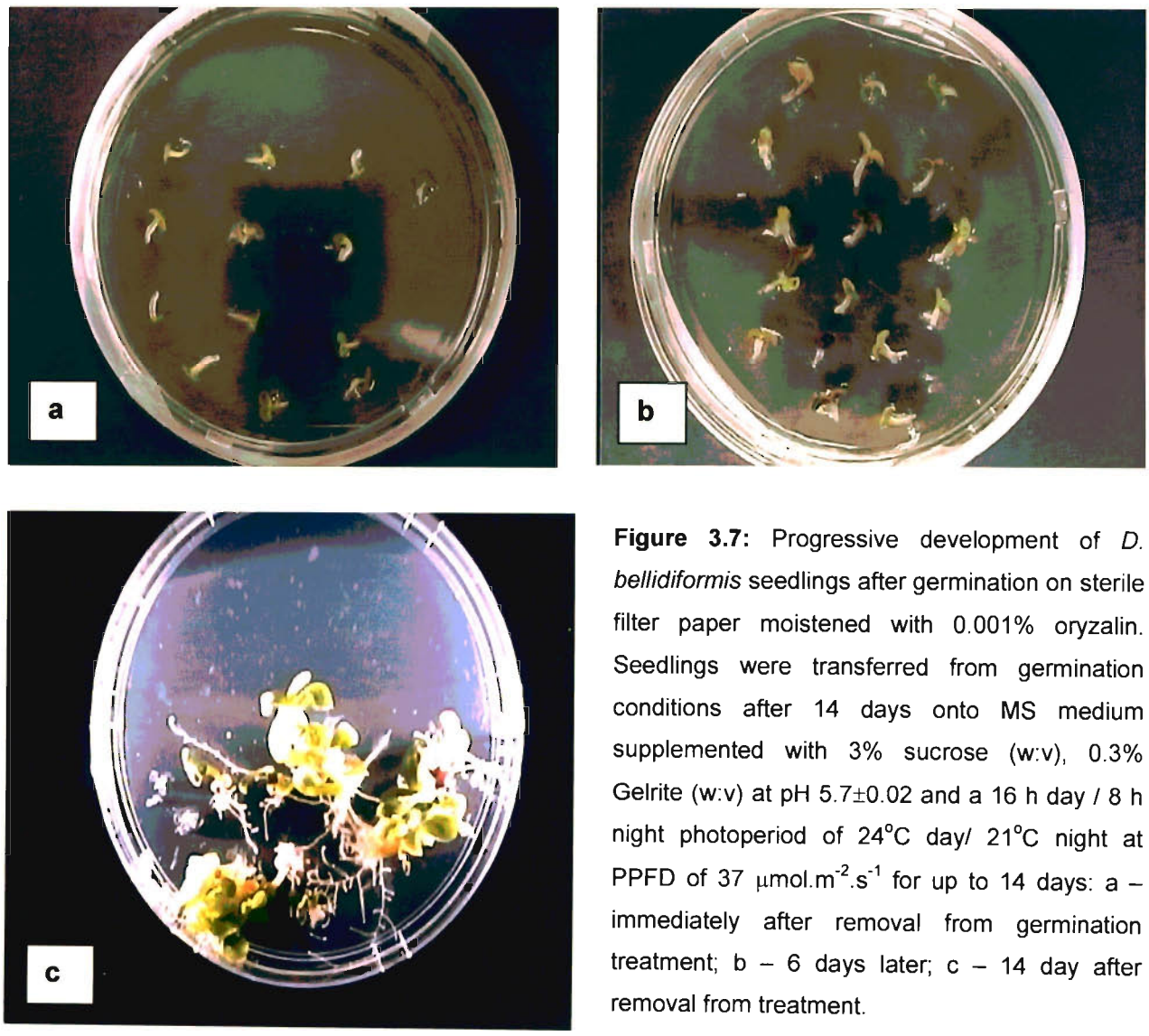


**Figure 3.7:** Progressive development of *D. bellidiformis* seedlings after germination on sterile filter paper moistened with 0.001% oryzalin. Seedlings were transferred from germination conditions after 14 days onto MS medium supplemented with 3% sucrose (w:v), 0.3% Gelrite (w:v) at pH 5.7±0.02 and a 16 h day / 8 h night photoperiod of 24°C day/ 21°C night at PPFD of 37 $\mu mol.m^{-2}.s^{-1}$ for up to 14 days: a – immediately after removal from germination treatment; b – 6 days later; c – 14 day after removal from treatment.

In view of the marked differences in growth and development of seedlings of *D. bellidiformis* in the presence of colchicine and oryzalin, light and electron-microscopy was used to characterize some of the tissue and cellular changes brought about by the different antimitotic treatments. Microscopical examination was made on the shoot and root apex of the germinated seedlings because of the known properties of interference of antimitotic compounds with microtubules and cell division. The cellular arrangement of meristematic cells from root apex isolated from seedlings germinated in the presence of 0.1 and 0.01% colchicine was similar to meristematic cells from the root tip of the control seedlings (Figure 3.8a), in contrast to seedlings from the control treatments, seedlings germinated in the presence of oryzalin was associated with an abnormal cellular arrangement in the root tip (Figure 3.8b). Light microscopy was used to establish the cellular arrangement in the root tip of seedlings germinated in the presence of oryzalin. The light microscopy analysis of the root sections from seedlings germinated in the presence of oryzalin indicates that tissue abnormalities underlies the abnormal root development observed when grown on sucrose-supplemented MS media (Figure 3.6 and 3.7). Due to the fine nature of the roots, and problems associated with orientation, sectioning and accurate identification of the meristematic region of the root, further studies were directed exclusively to the shoot tip region where orientation and the meristematic cell region were more readily identifiable. Meristematic cells isolated from the root apex of *D. bellidiformis* seedlings were difficult to identify, this was attributed to the extensive vacuolation and collapse observed. In contrast to meristematic cells from the root apex, cells isolated from the shoot apex of the same seedlings showed that there were differences in ultrastructure of cells isolated from the seedlings under the different experimental treatments considered.

There also appeared to be differences between the development of cells from shoot tip isolated from control seedlings that were germinated in the absence of antimitotic substances (Figure 3.9a), and in the presence of 0.1% colchicine (Figure 3.9b), 0.01% colchicine (Figure 3.9c) and oryzalin (Figure 3.9d). The

ultrastructure of cells from the shoot tip of the control seedlings (Figure 3.9a) had cytomatrix-rich regions, and sparse vacuolation. Shoot meristem cells isolated from seedlings germinated at 0.1% colchicine (Figure 3.9b) had many vacuolar inclusions, and the cytomatrix appeared fragmented. Cells isolated from the shoot meristematic region of seedlings germinated in the presence of 0.01% colchicine (Figure 3.9c) showed some cells that were highly vacuolated with a limited cytomatrix evident. Other cells appeared vacuolated but numerous starch grains were observed. Cells isolated from the shoot meristematic region of seedlings germinated in the presence of 0.01% and 0.001% oryzalin demonstrated a different morphology to colchicine treatments (Figure 3.9b and c) and the water controls (Figure 3.9a). A sparse cytomatrix was observed with evidence of vacuolation and extensive starch grain deposition in seedlings germinated in the presence of 0.01% colchicine (Figure 3.9c).

The shoot tip cells of the control *D. bellidiformis* seedlings were characterized by the appearance of large nuclei with prominent nucleoli. A sparse distribution of endoplasmic reticulum existed across the cytomatrix (Figure 3.10a and b). Mitochondria had limited membrane development and the plastids present in the cytomatrix had limited development of thylakoid stacking (Figure 3.10b and c). Dispersal of vacuoles was evident in the cytomatrix and vacuoles with occasional membraneous inclusions were also observed (Figure 3.10b).

The meristematic cells of the shoot apex isolated from seedlings germinated in the presence of 0.1% colchicine showed an ultrastructral condition indicative of a loss of cellular activity (Figures 3.11a-3.11c) accompanied by extensive vacuolation (Figure 3.11a), which restricted the cytomatrix to a narrow peripheral band (Figure 3.11a). Vacuolar engulfment of the cytomatrix was also observed (Figure 3.11a). An abnormal and irregular development of chloroplasts (Figure 3.11a) and mitochondria (Figure 3.11b) was seen. In contrast to meristematic cells isolated from shoot tip of seedlings germinated in

the presence of 0.1% colchicine, the ultrastructural condition of meristematic cells of the shoot tip of seedlings germinated in the presence of 0.01% colchicine appeared to show signs of enhanced intracellular activity (Figure 3.12a-c). The cytomatrix was restricted to a broad peripheral band by a large central vacuole (Figure 3.12a). There appeared to be an increase in starch grain volume compared with shoot meristematic cells isolated from seedlings germinated in the absence of antimitotic substances (Figure 3.12b). The nucleus was displaced towards the periphery of the cell showed the presence of some degree of hetrochromatin (Figure 3.12a). Development of organelles was observed, particularly mitochondria (Figure 3.12b). In addition, the inner membranes of plastids had developed well defined granal and stromal lamellae, typical of functional chloroplasts (Figure 3.12a). The chloroplasts also showed extensive starch deposition causing the compression of the inner lamellae towards the outer membrane of the organelle. Vacuoles with evidence of membranous inclusions were observed, as well as vacuoles with occasional inclusions of tannin-like bodies (Figure 3.12c).

The meristematic cells isolated from shoot tip of seedlings germinated in the presence of 0.01% oryzalin showed an ultrastructural condition indicative of deterioration (Figures 3.13a-c). The cytomatrix was restricted to a narrow peripheral band. In vacuolated cells, extensive cytomatrix inclusions were also observed within the vacuoles (Figure 3.13b), and a large number of tannin-like bodies inclusions were evident within the vacuoles (Figure 3.13c). The meristem cells were characterized by irregular wavy cell walls (Figure 3.13a), and the presence of somewhat oval nuclei (Figure 3.13a). Some mitochondria appeared lobed, and most showed electron-dense regions. Nuclei with prominent nucleoli were observed (Figure 3.13a) Abnormalities in the chloroplasts (Figure 3.13c) and internally undifferentiated mitochondria (Figure 3.13a) were evident.

In contrast to seedlings germinated at 0.01% oryzalin, seedlings germinated in the presence of 0.001% oryzalin showed a less disturbed ultrastructural appearance (Figures 3.14a-c). The ultrastructure of shoot meristematic cells showed evidence of enhanced meristematic activity, typified by well-defined endomembranous systems such as Golgi bodies and ER, distributed extensively throughout the cytomatrix (Figure 3.14a). The cytomatrix was typical of meristematic cells. Undifferentiated plastids, in addition to developing mitochondria, were observed (Figure 3.14b) Chloroplasts with starch deposits were evident (Fig. 3.14b). Occasional vacuoles with membranous inclusions were observed (Fig. 3.14c). The nucleoli were prominent, a typical feature in meristematic cells (Figure 3.14b).

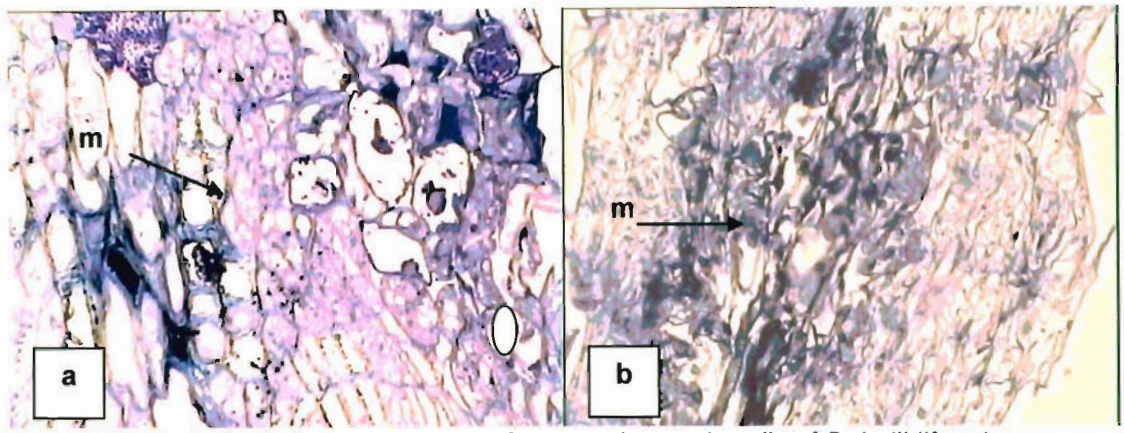


**Figure 3.8:** Typical cellular arrangement of root meristematic cells of *D. bellidiformis* seedlings: (a) control root tip, and (b) seedlings germinated in the presence of oryzalin , m – meristematic region [x25].

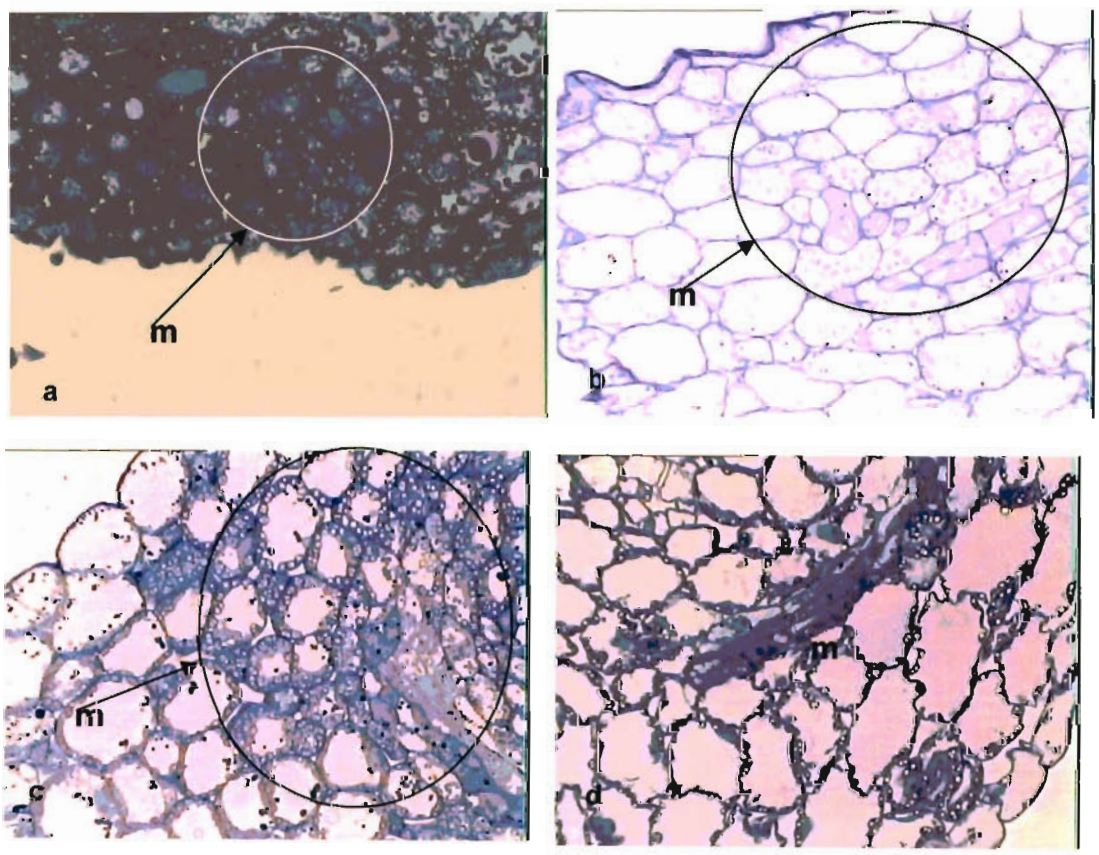


**Figure 3.9:** Cellular arrangement of shoot meristematic cells of *D. bellidiformis* from seedlings germinated in the presence of antimitotic substances: (a) control; (b) 0.1% colchicine; (c) 0.01% colchicine; (d) oryzalin, m – meristem region [x50].

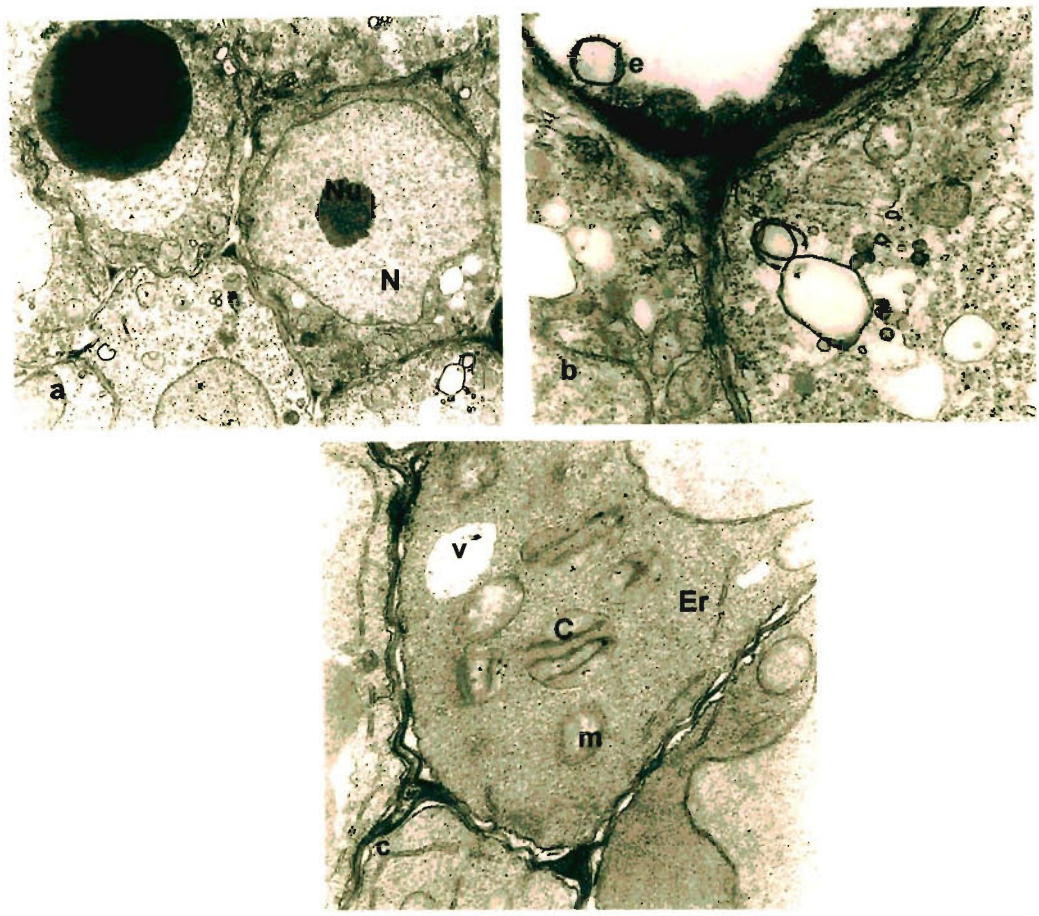


**Figure 3.10a-c:** The ultrastructure of meristematic cells from the shoot tip region of *D. Bellidiformis* seedlings germinated in the presence of sterile distilled water. Meristematic cells of the shoot tip were characterized by nuclei that diplayed a prominent nucleoli (Figure 3.10a [x5000]). These cells were also characterized by developing chloroplasts and mitochondria (Figure 3.10c [x12000]). Vacuoles also exhibited a smaller number of membranous inclusions (Figure 3.10b [x12000]). An endomembrane system was evident, and endoplasmic reticulum was observed as short profiles (Figure 3.10c).*

*N, Nucleus; Nu, Nucleolus; C, chloroplast; Er, endoplasmic reticulum; m, mitochondrion; v, vacuole; e, membranous inclusions

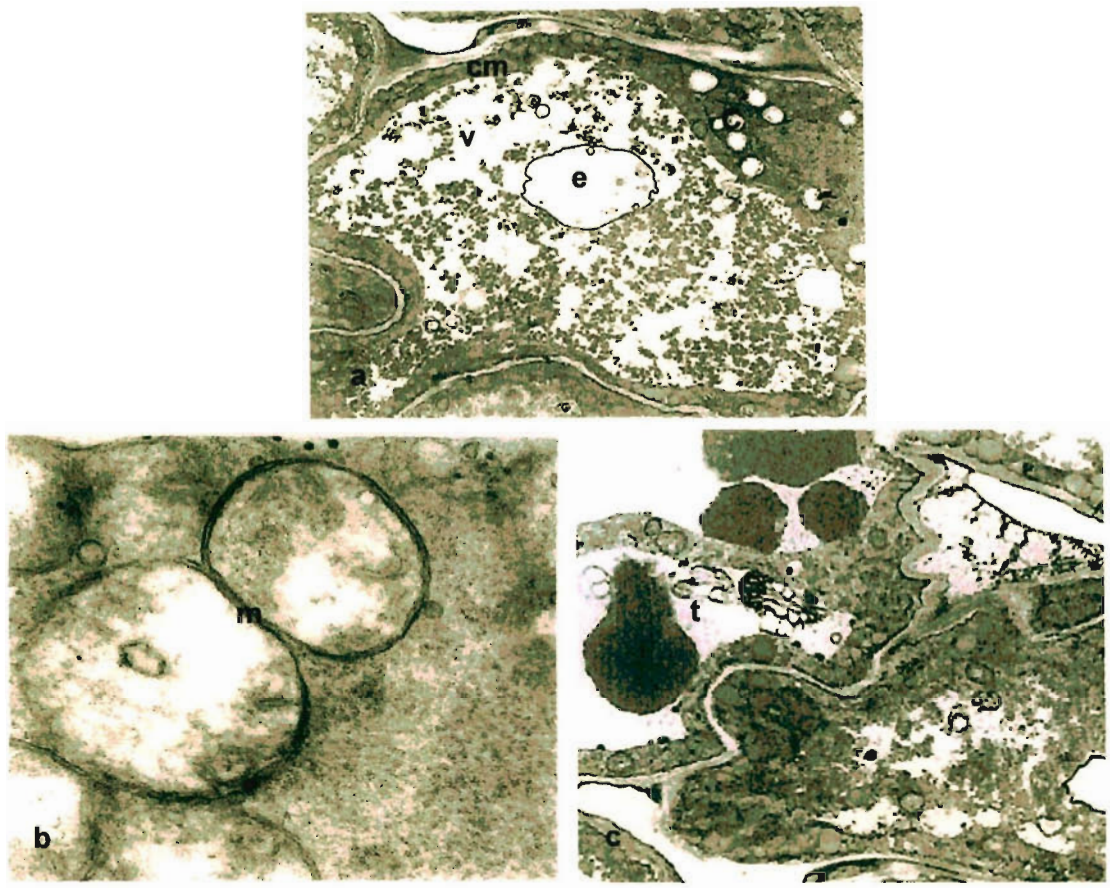


**Figure 3.11a-c:** The ultrastructure of meristematic cells from the shoot tip of *D. bellidiformis* seedlings germinated in the presence of 0.1% colchicine. Meristematic cells of the shoot tip were characterized by extensive vacuolation that restricted the cytomatrix to a narrow peripheral band (Figure 3.11a [x3000]). Cytomatrix engulfment by vacuoles was also observed (Figure 3.11a). Irregular chloroplast development was observed and limited starch deposition was evident (Figure 3.11a). Vacuoles also exhibited membranous inclusions (Figure 3.11a). Mitochondria showed limited internal membranes (Figure 3.11b [x60 000]). Vacuoles showed extensive tannin-like body inclusions (Figure 3.11c [x3000]).*

*C, chloroplast; cm, cytomatrix; m, mitochondrion; v, vacuole; e, membranous inclusions; t, tannin-like bodies

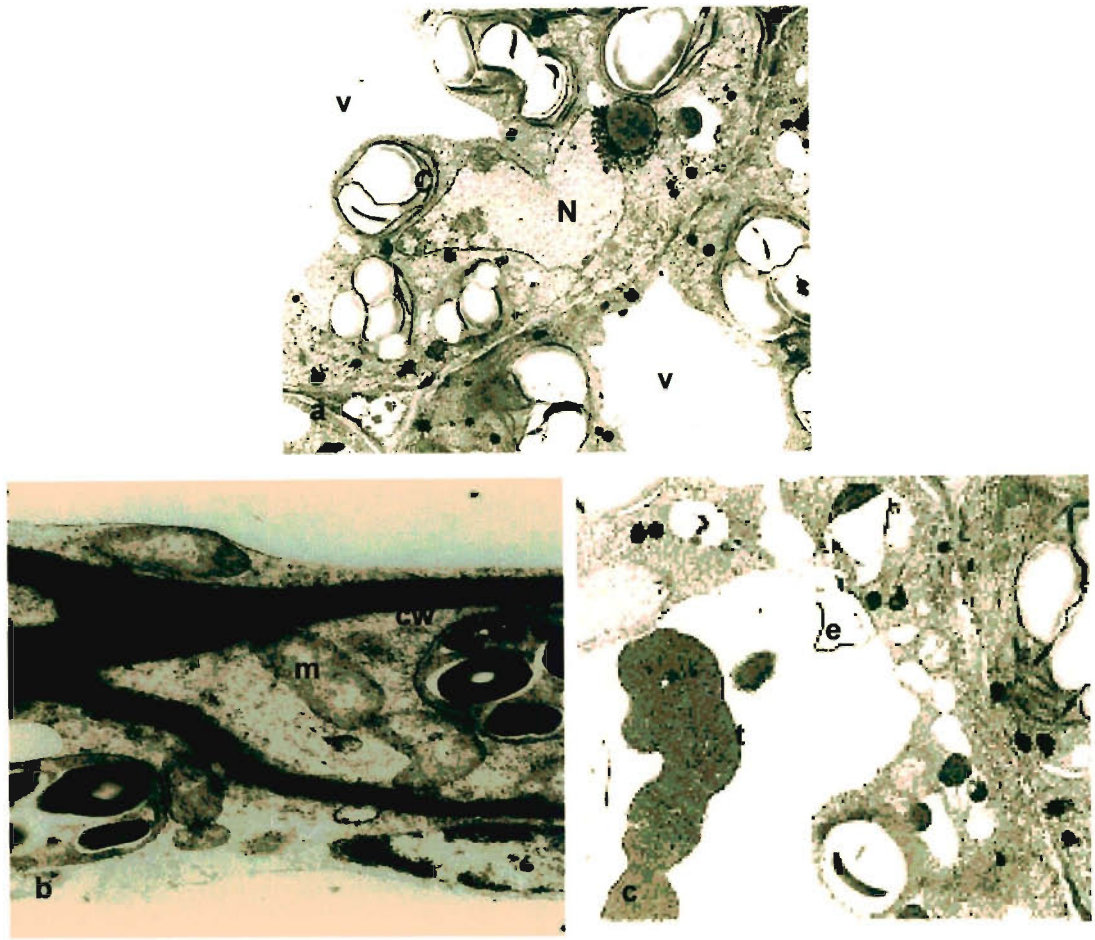


**Figure 3.12a-c:** The ultrastructure of meristematic cells from the shoot tip region of *D. bellidiformis* seedlings germinated in the presence of 0.01% colchicine. Meristematic cells of the shoot tip were characterized by a large degree of vacuolation and nuclei that displayed some hetrochromatin (Figure 3.12a [x4000]). These cells were also characterized by extensive starch deposition within the chloroplast (Figures 3.12a and 3.12b[x8000]). Undifferentiated mitochondria were evident (Figure 3.12b). Vacuoles also exhibited occasional inclusions of tannin-like bodies and membranous inclusions (Figure 3.12c [x4000]).*

*N, Nucleus; C, chloroplast; m, mitochondrion; v, vacuole; cw, cell wall; s, starch deposition; e, membranous inclusions; t, tannin-like bodies

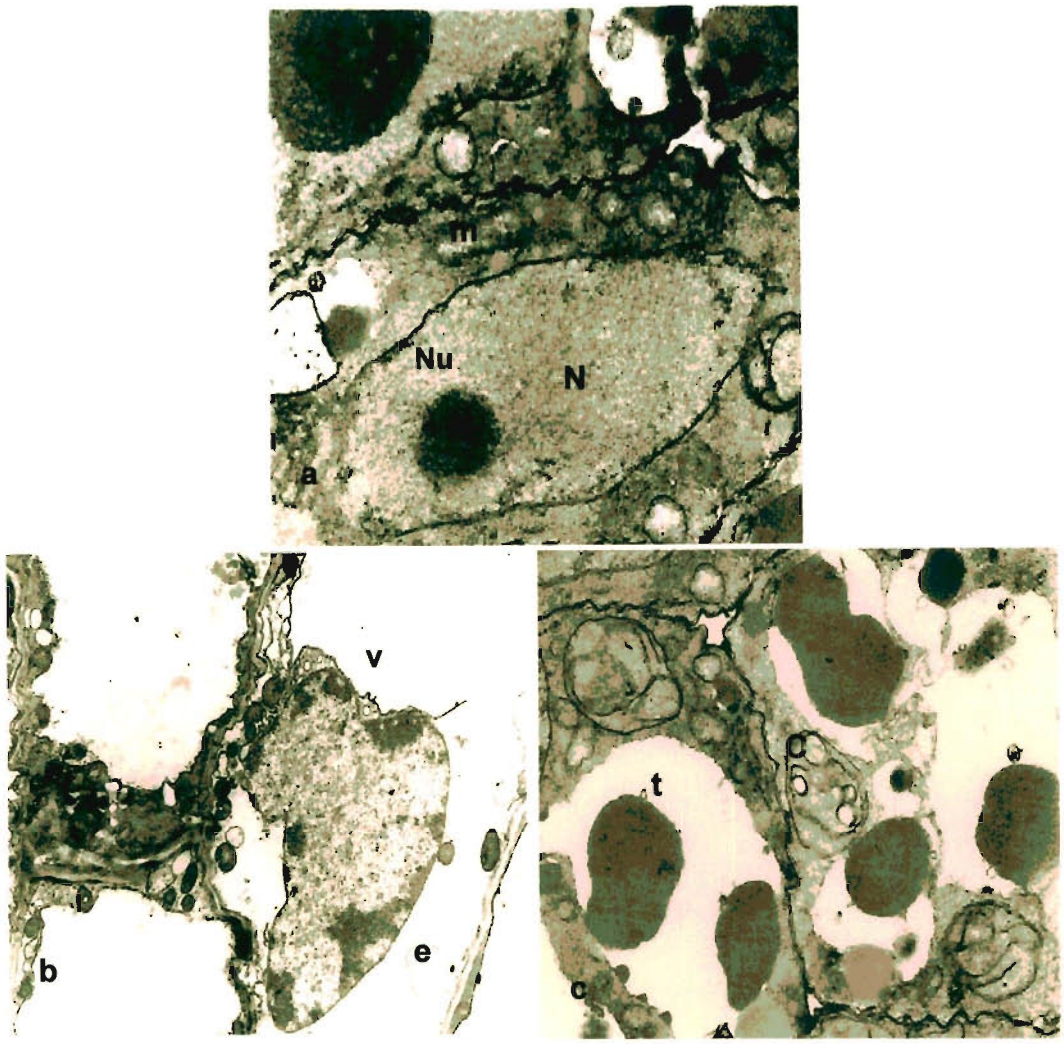


**Figure 3.13a-c:** The ultrastructure of meristematic cells from the shoot tip region of *D. bellidiformis* seedlings germinated in the presence of 0.01% oryzalin. Meristematic cells of the shoot tip were characterized by a large degree of vacuolation (Figure 3.13b [x2500]) and nuclei that displayed prominent nucleoli (Figure 3.13a [x4000]). Vacuoles also had a large degree of inclusions of tannin-like bodies (Figure 3.13c [x4000]), membranous and cytomatrix inclusions (Figure 3.13b [x4000]). Chloroplasts exhibited abnormal internal membrane formation (Figure 3.13c), internal membrane formation within mitochondria were also irregular (Figure 3.13a)*

*N, Nucleus; Nu, Nucleolus; C, chloroplast; m, mitochondrion; v, vacuole e, membranous inclusions; t, tannin-like body

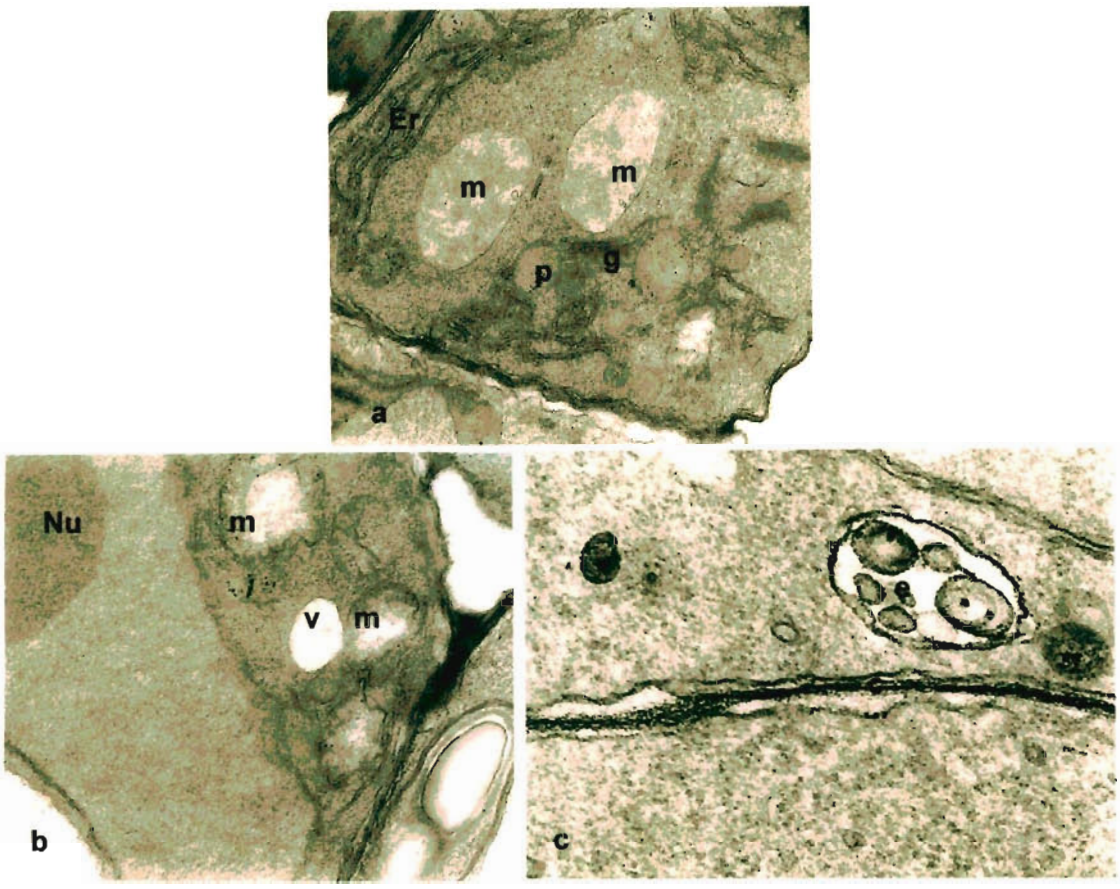


**Figure 3.14a-c:** The ultrastructure of meristematic cells from the shoot tip region of *D. bellidiformis* seedlings germinated in the presence of 0.001% oryzalin. Endoplasmic reticulum and Golgi bodies were evident in the meristematic cells of the shoot tip (Figure 3.14a [x15000]). Nuclei displayed prominent nucleoli (Figure 3.14b [x15000]). There was some degree of starch deposition within the chloroplast (Figure 3.14b). Mitochondria with well-developed cristae were evident (Figure 3.14a). Vacuoles were observed (Figure 3.14b), and exhibited occasional membranous inclusions (Figure 3.14c [x40000]). Undifferentiated plastids were also evident (Figure 3.14c).*

*Nu, nucleolus; Er, endoplasmic reticulum; g, Golgi body; m, mitochondrion; v, vacuole; e, membranous inclusions; p, plastids

## 3.3 RESPONSE TO *IN VITRO* POLYPLOIDIZATION TREATMENT AND SUBSEQUENT *IN VITRO* REGENERATION

Micro-shoots of *D. bellidiformis* and *M. whiteii* were cultured for 48 h in a liquid sucrose-supplemented MS medium containing antimitotic substances and thereafter cultured for several weeks on a solid sucrose-supplemented MS medium without any antimitotic substances. A number of practical constraints associated with *in vitro* plant culture systems limited the successful regeneration of treated explants. There are various problems encountered during the manipulation of plant material in tissue culture. In the present study several of the problems that limit successful regeneration of plant tissue culture were encountered, these included hyperhydricity, phenolics exudation viewed on the top of the medium, necrosis and contamination, and was observed in both *D. bellidiformis* (Figure 3.15b – e) and *M. whiteii* (Figure 3.16 b – e). These limiting factors were identified and various procedures were implemented to reduce the effect of these problems on regeneration of *in vitro* polyploidized plant material.

In a preliminary investigation it was observed that *D. bellidiformis* micro-shoots (Figure 3.15a) grew best at a 16 h day/8 h night photoperiod at 27°C /21°C, respectively and PPFD of 66 $\mu mol.m^{-2}.s^{-1}$ while *M. whiteii* micro-shoots (Figure 3.16a) grew best at a 16 h day/8 h night photoperiod of 24°C /21°C, respectively and PPFD of 37 $\mu mol.m^{-2}.s^{-1}$. These parameters were therefore used in the current investigation when micro-shoots of these species were cultured *in vitro*.

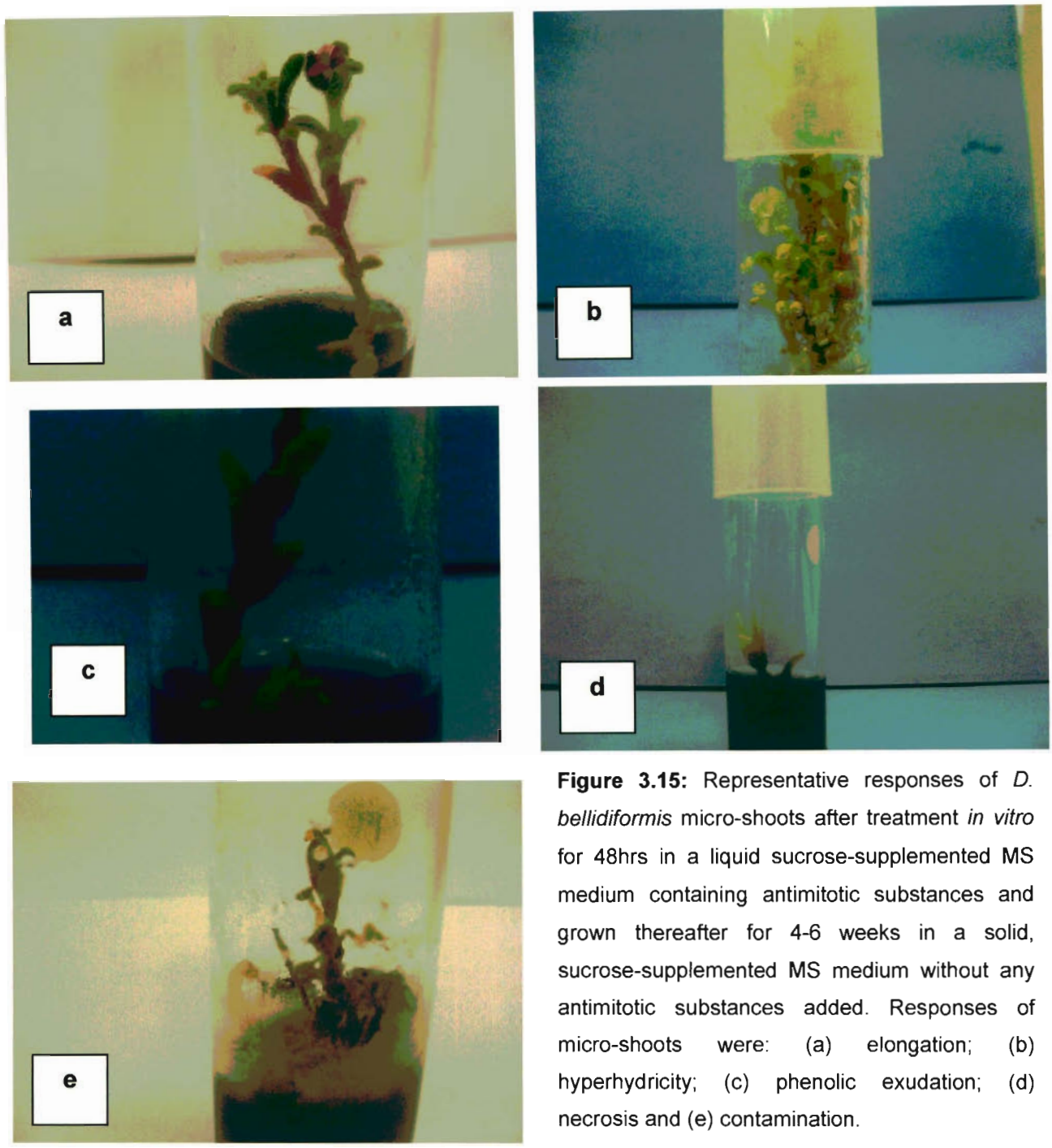


**Figure 3.15:** Representative responses of *D. bellidiformis* micro-shoots after treatment *in vitro* for 48hrs in a liquid sucrose-supplemented MS medium containing antimitotic substances and grown thereafter for 4-6 weeks in a solid, sucrose-supplemented MS medium without any antimitotic substances added. Responses of micro-shoots were: (a) elongation; (b) hyperhydricity; (c) phenolic exudation; (d) necrosis and (e) contamination.

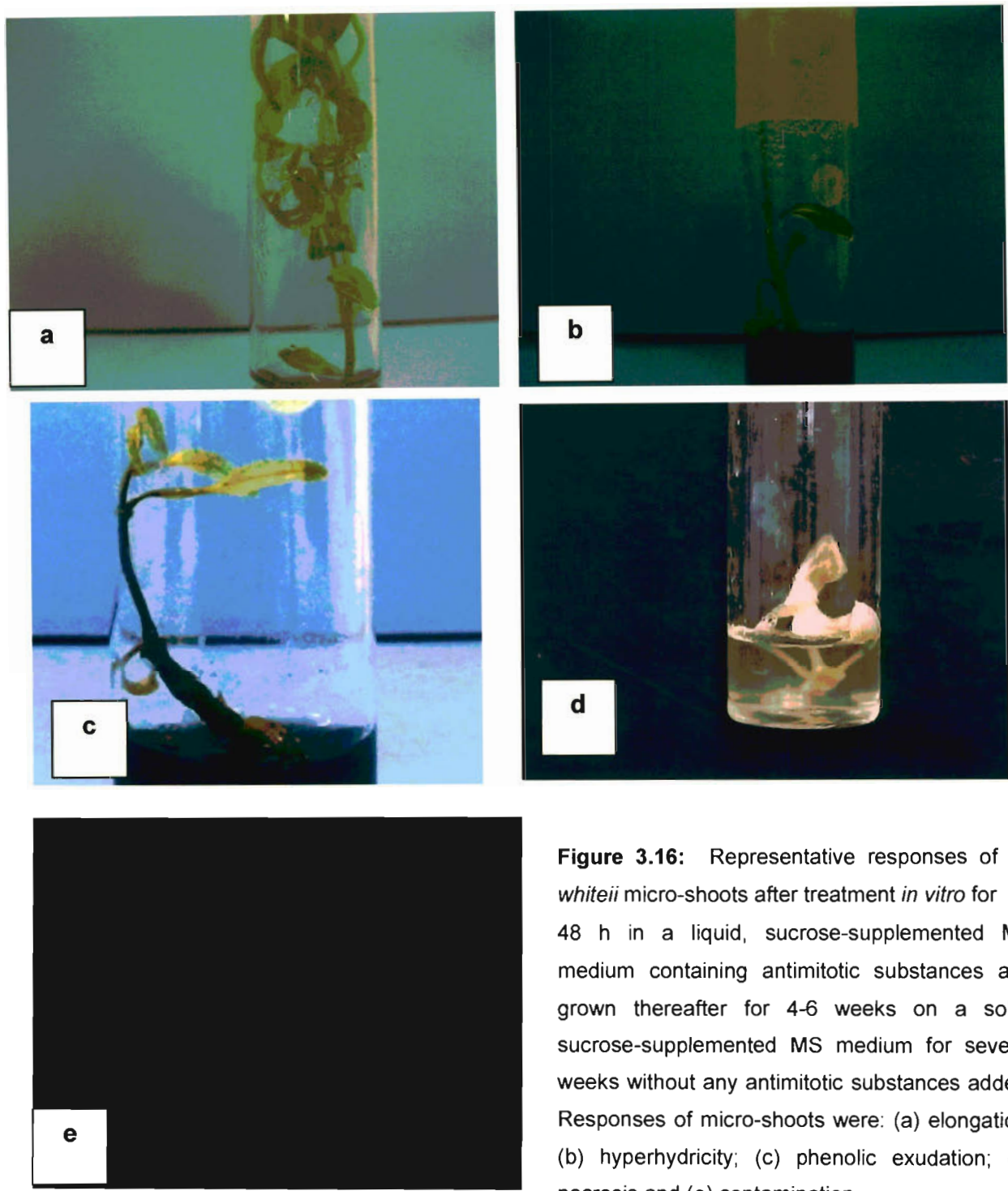


**Figure 3.16:** Representative responses of *M. whiteii* micro-shoots after treatment *in vitro* for 48 h in a liquid, sucrose-supplemented MS medium containing antimitotic substances and grown thereafter for 4-6 weeks on a solid, sucrose-supplemented MS medium for several weeks without any antimitotic substances added. Responses of micro-shoots were: (a) elongation; (b) hyperhydricity; (c) phenolic exudation; (d) necrosis and (e) contamination.

In the initial attempt to culture the treated micro-shoots, the explants treated with the antimitotic substances were cultured on a solid sucrose-supplemented MS medium under high relative humidity using a technique practiced at the ARC lab in Nelspruit, which involves sealing the culture tubes with parafilm (Hannweg and Vos, pers. comm.).

Micro-shoots of *D. bellidiformis* that were treated with antimitotic substances for 48 h and subsequently cultured on solid sucrose-supplemented MS medium for 6 weeks under high humidity conditions showed elongation of micro-shoots in all treatments with the two antimitotic substances at the two concentrations tested (Table 3.3). Although elongation was evident, a large number of micro-shoots were lost to contamination under all treatment conditions. Necrosis was observed in many micro-shoots that were treated as controls. Only two treatments showed signs of phenolic exudation: micro-shoots that were initially treated with 0.01% colchicine or 0.001% oryzalin (Table 3.3). There was no hyperhydricity observed in control micro-shoots or even in those that were initially treated with 0.001% oryzalin. In some instances it was evident that although elongation was observed, necrosis, contamination, hyperhydricity and phenolics exudation were evident in the micro-shoots as well (Table 3.3).

**Table 3.3:** Categories of responses of *D. bellidiformis* micro-shoots after six weeks of culture on MS medium supplemented with 3% sucrose and 0.3% Gelrite at a pH of 5.7±0.02.

| Treatment | Elongation (%) | Hyperhydricity (%) | Phenolic Exudation (%) | Necrosis (%) | Contamination (%) |
|---|---|---|---|---|---|
| Control | 30 | 0 | 0 | 95 | 75 |
| 0.1% Colchicine | 35 | 25 | 0 | 0 | 65 |
| 0.01% Colchicine | 40 | 15 | 5 | 0 | 70 |
| 0.01% Oryzalin | 20 | 0 | 0 | 20 | 80 |
| 0.001% Oryzalin | 40 | 15 | 10 | 0 | 65 |

Micro-shoots were previously treated with a 48h immersion in liquid MS medium supplemented with 3% sucrose at a pH of 7.00±0.02, containing 0.1% or 0.01% colchicine and 0.01% or 0.001% oryzalin and at a 16 h day/8 h night photoperiod at 27°C /21°C, respectively and PPFD of 66 $\mu mol.m^{-2}.s^{-1}$. Treated micro-shoots were contained in culture tubes sealed with Parafilm. Values represent mean of 20 replicates for each treatment.

Micro-shoots of *M. whiteii* treated with the two different antimitotic compounds at two different concentrations, responded differently when they were transferred on to sucrose-supplemented MS medium at a pH of 5.7±0.02, these cultures were also maintained at high humidity and reduced ventilation by sealing the caps to the culture tubes with Parafilm. After 6 weeks elongation was observed for all experimental treatments (Table 3.4). Hyperhydricity, phenolics exudation, necrosis and contamination were also evident in all treatments. Contamination was observed for *M. whiteii* explants in all experimental conditions, and was comparable to contamination recorded for *D. bellidiformis* cultured under similar conditions. Although necrosis was observed for all experimental treatments, a comparatively higher number of micro-shoots initially treated with 0.1% colchicine were lost to necrosis (70%).

**Table 3.4:** Categories of responses of *M. whiteii* micro-shoots after six weeks of culture on MS medium supplemented with 3% sucrose and 0.3% Gelrite at a pH of 5.7±0.02.

| Treatment | Elongation (%) | Hyperydricity (%) | Phenolic Exudation (%) | Necrosis (%) | Contamination (%) |
|---|---|---|---|---|---|
| Control | 55 | 15 | 20 | 25 | 20 |
| 0.1% Colchicine | 30 | 30 | 15 | 70 | 5 |
| 0.01% Colchicine | 75 | 15 | 15 | 35 | 10 |
| 0.01% Oryzalin | 60 | 5 | 10 | 40 | 5 |
| 0.001% Oryzalin | 55 | 5 | 30 | 45 | 10 |

The micro-shoots were previously treated with a 48 h immersion in liquid MS medium supplemented with 3% sucrose at a pH of 7.00±0.02, containing 0.1% or 0.01% colchicine and 0.01% or 0.001% oryzalin and at a 16 h day/8 h night photoperiod at 24°C /21°C, respectively and PPFD of 37 $\mu mol.m^{-2}.s^{-1}$. Treated micro-shoots were contained in culture tubes sealed with Parafilm. Values represent the mean of 20 replicates for each treatment.

Contamination, hyperhydricity, necrosis and phenolics exudation reduced the chances of survival of micro-shoots of both *D. bellidiformis* and *M. whiteii* treated with antimitotic substances. Therefore, in an attempt to reduce the effect of all these phenomena during plant tissue culture, it was necessary to reduce the humidity and increase the ventilation within the culture tubes, which was achieved by not sealing the caps with parafilm.

Elongation was evident for a greater or lesser percentage of micro-shoots of *D. bellidiformis* initially treated with 0.1% or 0.01% colchicines, and 0.01% or 0.001% oryzalin and thereafter cultured for 6 weeks on solid sucrose-supplemented MS medium, at a reduced humidity (Table 3.5). Although many micro-shoots were lost to contamination, no phenolics exudation, hyperhydricity and necrosis was evident (Table 3.5), compared with micro-shoots that were cultured at a higher humidity (Table 3.3). Only 10% of the micro-shoots initially treated for 48 h with 0.1% colchicine were lost to contamination, while a 60%

loss was observed for controls (Table 3.5). Although phenolics exudation, hyperhydricity and necrosis appeared to be completely eliminated, the rest of the micro-shoots were lost to contamination. This loss was relatively higher in comparison with *D. bellidiformis* micro-shoots treated with the same antimitotic substances and cultured on the same medium but at a higher relative humidity (Table 3.3).

**Table 3.5:** Categories of response of *D. bellidiformis* micro-shoots after six weeks of culture on MS medium supplemented with 3% sucrose (w:v) and 0.3% Gelrite at a pH of 5.7±0.02.

| Treatment | Elongation (%) | Hyperhydricity (%) | Phenolic Exudation (%) | Necrosis (%) | Contamination (%) |
|---|---|---|---|---|---|
| Control | 40 | 0 | 0 | 0 | 60 |
| 0.1% Colchicine | 90 | 0 | 0 | 0 | 10 |
| 0.01% Colchicine | 75 | 0 | 0 | 0 | 25 |
| 0.01% Oryzalin | 45 | 0 | 0 | 0 | 55 |
| 0.001% Oryzalin | 65 | 0 | 0 | 0 | 35 |

Micro-shoots were previously treated with a 48 h immersion in liquid MS medium supplemented with 3% sucrose (w:v) at a pH of 7.00±0.02, containing 0.1% or 0.01% colchicine (w:v) and 0.01% or 0.001% oryzalin (w:v) and at a 16 h day/8 h night photoperiod at 27°C /21°C, respectively and PPFD of 66 $\mu mol.m^{-2}.s^{-1}$. Values represent the mean of 20 replicates for each treatment.

The results of the experimental treatment reported in Table 3.6 represent the categories of responses of *M. whiteii* micro-shoots treated with colchicine or oryzalin 48hs and thereafter transferred onto a solid sucrose-supplemented MS medium. The micro-shoots were maintained for 6 weeks under a 16 h day/ 8 h night photoperiod at 24°C/21°C, respectively and PPFD at 37 $\mu mol.m^{-2}.s^{-1}$. In

this instance the humidity in the individual culture tubes were reduced by not sealing the culture tubes with Parafilm.

Elongation was observed in micro-shoots from all antimitotic treatments, and contamination and necrosis were evident in all cases (Table 3.6). In contrast to results obtained when the culture tubes were sealed with Parafilm (Table 3.4), hyperhydricity (10%) was observed only in micro-shoots treated with 0.001% oryzalin (Table 3.6). Phenolics exudation was evident in micro-shoots from all treatments except in those micro-shoots initially treated with 0.01% colchicine. Necrosis and contamination was evident for all treatment conditions (Table 3.6), this was also observed in the micro-shoots that were maintained at a high humidity (Table 3.4).

**Table 3.6:** Categories of responses of *M. whiteii* micro-shoots after six weeks of culture on MS medium supplemented with 3% sucrose and 0.3% Gelrite at a pH of 5.7±0.02.

| Treatment | Elongation (%) | Hyperhydricity (%) | Phenolic Exudation (%) | Necrosis (%) | Contamination (%) |
|---|---|---|---|---|---|
| Control | 65 | 0 | 35 | 30 | 20 |
| 0.1% Colchicine | 30 | 0 | 15 | 45 | 15 |
| 0.01% Colchicine | 60 | 0 | 0 | 10 | 20 |
| 0.01% Oryzalin | 20 | 0 | 20 | 35 | 15 |
| 0.001% Oryzalin | 30 | 10 | 10 | 20 | 10 |

The micro-shoots were previously treated with a 48 h immersion in liquid MS medium supplemented with 3% sucrose at a pH of 7.00±0.02, containing 0.1% or 0.01% colchicine and 0.01% or 0.001% oryzalin and at 16 h day/ 8 h night photoperiod of 24°C/21°C, respectively and PPFD at 37 $\mu mol.m^{-2}.s^{-1}$. Values represent the mean of 20 replicates for each treatment.

In an attempt to reduce phenolics exudation and necrosis on treated micro-micro-shoots, they were transferred onto sucrose-supplemented MS medium, incorporating 0.2% activated charcoal. Micro-shoots of *D. bellidiformis* treated for 48 h with antimitotic substances and thereafter cultured on a sucrose-supplemented solid MS medium containing 0.2% activated charcoal for a reduced culture period of four weeks, showed poor growth (data not presented)

In contrast to results obtained with *D. bellidiformis*, micro-shoots of *M. whiteii* treated for 48 h with antimitotic substances and then cultured on solid sucrose-supplemented MS medium containing activated charcoal, elongation was evident in many micro-shoot, while fewer micro-shoots showed phenolics exudation, contamination and necrosis (Table 3.7). All micro-shoots of *M. whiteii* initially treated for 48 h with 0.001% oryzalin showed evidence of elongation, as did 90% of the control micro-shoots and micro-shoots treated with 0.01% colchicine and 0.01% oryzalin. Elongation was also evident in 80% of the micro-shoots that were initially treated with 0.1% colchicine. However, by the end of the six week culture period, 30% of the micro-shoots were lost to necrosis. Necrosis also became evident in those micro-shoots that showed some initial growth.

Although all the micro-shoots treated initially with 0.001% oryzalin showed evidence elongation, there was also evidence of hyperhydricity (5%) and phenolics exudation (25%) in some of the micro-shoots. While 10% of the control treated micro-shoots and 5% of the micro-shoots treated initially with 0.1% colchicine and 0.01% oryzalin were lost to contamination, 10% of the micro-shoots treated with 0.1% or 0.01% colchicine, while 5% of the micro-shoots initially treated with 0.01% or 0.001% oryzalin showed signs of hyperhydricity (Table 3.7).

**Table 3.7:** Categories of responses of *M. whiteii* micro-shoots after 6 weeks of culture on MS medium supplemented with 3% sucrose, 0.2% activated charcoal and 0.3% Gelrite at a pH of 5.7±0.02.

| Treatment | Elongation (%) | Hyperhydricity (%) | Phenolic Exudation (%) | Necrosis (%) | Contamination (%) |
|---|---|---|---|---|---|
| Control | 90 | 0 | 0 | 5 | 10 |
| 0.1% Colchicine | 80 | 10 | 0 | 30 | 5 |
| 0.01% Colchicine | 90 | 10 | 5 | 10 | 0 |
| 0.01% Oryzalin | 90 | 5 | 10 | 5 | 5 |
| 0.001% Oryzalin | 100 | 5 | 25 | 0 | 0 |

The micro-shoots were previously treated with a 48 h immersion in liquid MS supplemented with 3% sucrose at a pH of 7.00±0.02, containing 0.1% or 0.01% colchicine and 0.01% or 0.001% oryzalin and at a 16 h day/8 h night photoperiod of 24°C/21°C, respectively and PPFD at 37 $\mu mol.m^{-2}.s^{-1}$. Values represent the mean of 20 replicates for each treatment.

In another attempt to reduce the effect of hyperhydricity on growth, micro-shoots of *M. whiteii* treated for 48 h in sucrose-supplemented MS medium containing antimitotic substances were cultured on solid sucrose-supplemented MS medium containing activated charcoal for a culture period reduced from 6 weeks to four weeks (Table 3.8). All control micro-shoots showed evidence of elongation as did 100% of the micro-shoots cultured initially in the presence of 0.01% colchicine (Table 3.8). There was no evidence of necrosis in any of the treatments. Contamination was observed only in oryzalin-treated micro-shoots. The number of explants showing hyperhydricity was low and evident only in control micro-shoots and those treated with 0.01% oryzalin (Table. 3.8). Exudation of phenolics was evident in micro-shoots from all experimental treatments except those that were initially treated with 0.01% colchicine.

**Table 3.8:** Categories of responses of *M. whiteii* micro-shoots after four weeks of culture on MS medium supplemented with 3% sucrose (w:v), 0.2% activated charcoal (w:v) and 0.3% Gelrite at a pH of 5.7±0.02.

| Treatment | Elongation (%) | Hyperhydricity (%) | Phenolic Exudation (%) | Necrosis (%) | Contamination (%) |
|---|---|---|---|---|---|
| Control | 100 | 5 | 5 | 0 | 0 |
| 0.1% Colchicine | 65 | 0 | 45 | 0 | 0 |
| 0.01% Colchicine | 100 | 0 | 0 | 0 | 0 |
| 0.01% Oryzalin | 85 | 5 | 15 | 0 | 10 |
| 0.001% Oryzalin | 70 | 0 | 5 | 0 | 35 |

The micro-shoots were previously treated with a 48 h immersion in liquid MS supplemented with 3% sucrose (w:v) at a pH of 7.00±0.02, containing 0.1% or 0.01% colchicine (w:v) and 0.01% or 0.001% oryzalin (w:v) and at a 16 h day/8 h night photoperiod of 24°C/21°C, respectively and PPFD at 37 $\mu mol.m^{-2}.s^{-1}$. Values represent the mean of 20 replicates for each treatment.

## 3.4 PLOIDY ANALYSIS

As a result of pure survival of *D. bellidiformis* micro-shoots in culture following treatment with antirnitotic substances (Tables 3.3 and 3.5) there were an insufficient number of plantlets that survived the elongation step. Therefore leaf samples from treated micro-shoots of *D. bellidiformis* were not available for assessment of the ploidy status of treated micro-shoots of *D. bellidiformis*. However, *Mondia whiteii* micro-shoots survived best in culture for four weeks on a sucrose-supplemented MS medium containing activated charcoal from all elongation treatments considered (Table 3.7). Therefore sufficient plantlets were available of this species to asses its ploidy status.

The ploidy status was assessed by flow cytometry on leaves of *M. whiteii* micro-shoots initially treated by a 48 h immersion in liquid sucrose-supplemented MS at a pH of 7.00±0.02, containing 0.1% or 0.01% colchicine and 0.01% or

0.001% oryzalin. The treated explants were thereafter cultured for four weeks on solid sucrose-supplemented MS containing 0.2% activated charcoal at a pH of 5.7±0.02 and at a 16 h day/8 h night photoperiod at 24°C/21°C, respectively and a PPFD of 37 $\mu mol.m^{-2}.s^{-1}$. Only micro-shoots initially treated with 0.01% oryzalin were identified as polyploids (Table 3.9), while all those treated initially with 0.001% oryzalin remained as diploids. Mixaploids were also identified in explants treated with 0.01% oryzalin, 0.1% and 0.01% colchicine (Table 3.9).

**Table 3.9:** Ploidy level of leaves of *M. whiteii* from micro-shoots after treatment with the antimitotic substances, oryzalin and colchicine in liquid sucrose-supplemented MS medium.

| Treatment | % Diploids (2n) | % Polyploids (3n) | % Mixaploid |
|---|---|---|---|
| 0.1% colchicine | 95 | 0 | 5 |
| 0.01% colchicine | 90 | 0 | 10 |
| 0.01% oryzalin | 70 | 10 | 20 |
| 0.001% oryzalin | 100 | 0 | 0 |

Prior to ploidy analysis micro-shoots were cultured on MS medium supplemented with 3% sucrose, 0.2% activated charcoal and 0.3% Gelrite at a pH of 5.7±0.02 for four weeks at a 16 h day/ 8 h night photoperiod of 24°C/21°C, respectively and PPFD at 37 $\mu mol.m^{-2}.s^{-1}$.

# CHAPTER FOUR
# DISCUSSION

## 4.1 ESTABLISHING A STERILIZATION TREATMENT

A principal objective of plant tissue culture is to produce plants that are completely free of contaminants. In micropropagation laboratories, stock plants from which explants are derived, are maintained under sterile conditions and disease free and routine sterilization procedures are implemented when the explant is transferred to *in vitro* conditions (Leifert and Cassells, 2001; Hartman *et al.*, 1997 and Torres, 1989). The status of explants obtained from plants of wild populations is unknown and therefore the sterilization procedures applied to these explants has to play a significant role in eliminating the persistent unnoticed contaminants. Surface sterilization of explants used for *in vitro* plant tissue culture is an important procedure to successfully achieve "healthy" disease free plant tissue cultures. Several sterilization procedures have been developed by a number of research and commercial laboratories. However this is in contrast to investigations that have reported persistent losses even though sterilization of the explants is routinely carried out (Leifert and Waites, 1990).

In the present study *D. bellidiformis* seeds purchased from McDonald Seeds and *M. whiteii* seeds purchased from Silverhill Seeds were used as initial starting material for the investigations that were carried out. The source of and the contamination status of the seeds were therefore unknown. Since explants used in the present investigation were seeds, it was assumed that the presence of a seed coat would have prevent the entry of any endogenous contaminants, therefore only a routine surface sterilization procedure was used to disinfect the outer surface of the seeds.

An attempt was made to surface sterilize the seed explants of *D. bellidiformis* and *M. whiteii* with a 1% NaOCl solution containing a drop of Tween 20. When this treatment was applied for 8min on *D. bellidiformis*, 75% of the seeds that germinated were free of any surface contamination (Table 3.1), however when

this treatment was applied for 8 min on *M. whiteii*, all the seeds germinated and showed no signs of surface contamination. Nodal cuttings were made of uncontaminated seedlings of both *D. bellidiformis* and *M. whiteii* and transferred onto multiplication medium. Enough *in vitro* shoots were derived from these explants for *in vitro* polyploidization experiments. Although these explants did not show any visible contamination when they were transferred onto the multiplication medium, contamination was evident in the micro-shoots after they were treated with antimitotic substances in a liquid sucrose supplemented MS medium for 48-hrs and thereafter transferred onto semi-solid sucrose supplemented MS medium.

Possible reasons for the persistence of contamination even though the surface of the seeds were disinfected include: poor sterile techniques applied when the micro-shoots were subcultured onto fresh media, or the presence of endogenous contaminants. If endogenous contaminants were present, attempts to disinfect the seeds only was inadequate to eliminate surface contaminants.

## 4.2 ATTEMPTS TO DETERMINE RESPONSES OF PLANT CELLS TO ANTIMITOTIC SUBSTANCES

The most frequently used method to efficiently induce polyploidy in several commercial plant species has been the *in vitro* treatment of plant material with antimitotic substances, particularly colchicine (Hancock, 1997). The concentration of colchicine necessary to induce polyploidy in plants is high and often the concentrations that are most efficient induce several phytotoxic responses. These responses include low fertility, failure to regenerate and extensive cell death. Another antimitotic substance, oryzalin has been considered to induce polyploidy in several plant species (Morejohn *et al.*, 1987). This antimitotic substance is favoured as it is less toxic to humans while it binds more efficiently with plant tubulin than does colchicine (Verhoeven *et al.*, 1990 and Morejohn *et al.*, 1987). Concentrations of oryzalin necessary to induce polyploidy in plants have been shown to be significantly lower than concentrations of colchicine necessary to induce polyploidy (Geoffriau *et al.*, 1997; Morejohn *et al.*, 1987 and van Tuyl *et al.*, 1992).

While ample evidence is available on the efficiency of these two compounds as polyploidization agents (Cohen and Yao, 1996, van Tuyl *et al.*, 1992), very little has been reported on the implications of these antimitotic compounds on growth, development and ultrastructure. Limited evidence is also available on the germination response of seeds to treatment with antimitotic substances. However, the morphological responses of plants following treatment with antimitotic substances and the characteristics of increased polyploidy have been well documented (van Tuyl *et al.*, 1992; Awoleye *et al.*, 1996 and Hancock, 1997).

### 4.2.1 Establishing the germination response to antimitotic substances

In the present study, attempts were made to establish the germination response of *D. bellidiformis* to treatment with two antimitotic substances (oryzalin and colchcicine). Results showed that germination of seeds *in vitro* were moderately affected by the treatments with 0.1% and 0.01% colchicine (Table 3.2), while a notable reduction in the germination response of seeds in the presence of the highest oryzalin concentration (0.01%) was observed. Although results have shown that germination in the presence of 0.1% and 0.01% colchicine and 0.001% oryzalin were relatively unaffected (Table 3.2), evidence of the morphological affects of the antimitotic substances only became apparent once the germinated seedlings were transferred to a sucrose supplemented MS medium. Most experiments using colchicine and oryzalin to induce polyploidy in plants have primarily focused on the efficiency of these compounds as agents to induce polyploidy (Wu and Mooney, 2002; Peterson *et al.*, 2003; Shao *et al.*, 2003 and Eeckhaut *et al.*, 2004).

It has been proposed previously that oryzalin at relatively lower concentrations is capable of producing similar responses in the meristematic cells treated with colchicine at much higher concentrations (Morejohn *et al.*, 1987). The results of the present study failed to support this observation, since differences in growth and development were observed in *D. bellidiformis* seedlings germinated in the presence of colchicine and oryzalin (Figure 3.1). While *D. bellidiformis* seedlings

that were treated with oryzalin were characterised by short, slow-growing roots that lacked root hairs (Figures 3.6 and 3.7), colchicine-treated seedlings were characterised by fast growing root systems that developed root hairs on the primary roots, and also showed shoot elongation (Figures 3.4 and 3.5).

### 4.2.2 Establishing the effects of antimitotic substances on cell ultrastructure

Most investigations concerning the effects of colchicine and oryzalin on plant development have focused primarily on the efficiency of these compounds to induce polyploidy (Väinölä, 2000; Peterson *et al.*, 2003 and Shao *et al.*, 2003), however, very little has been reported on the ultrastructural responses of plant cells to these antimitotic substances.

Microtubules are not only implicated in cell division but with various other physiological processes within the cell, microtubules are also an important component of the cytoskeleton (Gunning and Hardham, 1982 and Pickett-Heapes, 1974). As a consequence a block in microtubule activity will have a "cascading effect" on general cellular activity, and possibly lead to other, non-nuclear effects. A more detailed knowledge of the ultrastructural reponses to these antimitotic compounds is imperative, as it will allow for a more refined identification of concentrations of antimitotic compounds allowing successfully polyploidized organisms to reach maturity without inducing additional damaging changes to cellular activity.

In the current study, light-microscopy and transmission electron-microscopy have been used in an attempt to outline some of the ultrastructural responses of plant cells to treatments with colchicine and oryzalin. Light microscopy and observations on root meristematic tissue from oryzalin-treated seedlings showed severe meristematic tissue deterioration, cellular collapse and necrosis (Figure 3.8b). van Tuyl *et al.* (1992) reported that the oryzalin concentrations between 0.001% and 0.01% were less inhibiting to plant regeneration in experiments to induce polyploidy in *Nerine* and *Lillium*, but 0.01% and 0.001% concentration of oryzalin was also used in the present study. It is also important

to note that oryzalin, a dinitroanaline, is used as a herbicide (van Tuyl *et al.*, 1992), and it is therefore not surprising that it was responsible for the retarded root growth of the seedlings in the present investigation. The reduced root growth observed for oryzalin-treated seedlings (Figures 3.6and 3.7) and, the light microscopy observations of the root meristematic cells (Figure 3.8b), supports evidence previously proposed that oryzalin binds more readily with plant tubulin than does colchicine at the same concentration. The marked differences in growth and development observed in shoots treated with 0.01% oryzalin (Figure 3.6c) and colchicine (Figure 3.5c) also support evidence from previous studies of the different responses of plant cells to the two different antimitotic substances. The results from the present study failed to support the evidence of the regeneration potential observed by van Tuyl *et al.* (1992) after treatment with 0.01% oryzalin (Figure 3.6), suggesting that the concentration is more inhibitory to growth and regeneration in *D. bellidiformis*.

A study of the ultrastructure of meristematic cells from shoot tips of seedlings treated with the two different antimitotic substances, at the two different concentrations tested, revealed distinct differences. Abnormal development was observed in the mitochondria in meristematic cells from the shoot tip of seedlings germinated at 0.1% colchicine (Figure 3.11c) and 0.01% oryzalin (Figure 3.13a). Reports on colchicine induced polyploidy have shown that an increase in cell and organelle size to be a characteristic feature of induced polyploidy (Levin, 1983; Hassan *et al.*, 1991; Cohen and Yao, 1996 and Hancock, 1997). In the present study, screening for polyploidization of the treated seedlings was not carried out but there was clear evidence of the effects on organelle development, particularly with the mitochondria and chloroplasts (Figures 3.11, 3.12, 3.13 and 3.14). A feature of the cells isolated from treated seedlings, is the presence of apparent tannin-like bodies (Figures 3.12c and 3.13c), this possibly being indicative of a stress-induced response, by growth inhibition. Another unusual feature of the cell ultrastructure observed particularly with cells from shoot tips of seedlings treated with 0.1% colchicine, is the abnormal deterioration of the cytomatrix (Figure 3.11a), this could possibly be due to the action of the antimitotic substances on the microtubles of the cytoskeletal network (Gunning and Hardham, 1982 and Pickett-Heape, 1974).

This also suggests that if microtubules are the constituents of the cytoskeletal network and antimitotic substances are involved in inihibiting non-nuclear microtubules, then it is possible that this could account for the failure of a number of species to regenerate following *in vitro* treatment with colchicine (van Tuyl *et al.*, 1992 and Väinölä, 2000).

Evidence has been presented that mitochondrial and chloroplast division is controlled by a protein known as dynamin (Bleazard *et al.*, 1999 and Osteryoung *et al.*, 2000). Dynamin is also required for endocytosis, forming a collar on the outer surface of budding vesicles that acts to pinch the vesicles of the cell membrane (Hinshaw and Schmid, 1995 and McNevin *et al.*, 2000). Dynamin is reported to form rings and spirals resembling those formed by tubulin (Erikson, 2000). Mutations in the genes encoding for dynamin have been responsible for morphological abnormalities in the mitochondrion as defective severing of the outer, but not the inner membrane (Bleazard *et al.*, 1999). Electron microscopy of the cells treated with antimitotic agents in the current investigation have been consistent with results of the inhibition of mitochondrial fission previously reported by Bleazard *et al.* (1999). The involvement of dynamin in mitochondria and chloroplast division (Bleazard *et al.*, 1999; Osteryoung *et al.*, 2000) and the various ultrastructural changes accompanying treatment with antimitotic substances, provides scope for further detailed studies on organelle responses to antimitotic substances.

## 4.3 RESPONSES OF MICRO-SHOOTS TREATED WITH ANTIMITOTIC SUBSTANCES TO CULTURE CONDITIONS

Extensive work has been carried out on the *in vitro* polyploidization of several indigenous plant species as part of the efforts at the Agricultural Research Council to commercialize indigenous plants (Refer to Table 1.2, Chapter 1). While their investigations have considered the effect of the antimitotic substance used as an important aspect of *in vitro* polyploidization, evidence of the effect on subsequent *in vitro* growth of the treated plant material has been limited. There are various problems encountered when plants are regenerated

*in vitro* (Murashige, 1977; Linsey and Jones, 1989; Torres, 1989 and George, 1993). Factors that effect *in vitro* plant regeneration will ultimately effect the survival of prospective polyploids when *in vitro* polyploidization experiments are carried out. In the present study problems encountered with the *in vitro* regeneration included the onset of contamination, hyperhydricity, phenolic exudation and necrosis. Attempts were therefore made to reduce these responses.

The presence of internal pathogens in an explant may only become evident after several weeks of *in vitro* culture (Liefert and Waites, 1990), particularly when the explant is transferred onto different culture media (Hartman *et al.*, 1997). In the current investigation, *D. bellidiformis* micro-shoots and *M. whiteii* micro-shoots were derived from nodal cuttings of seedlings germinated *in vitro* on a solid sucrose-supplemented MS medium. Explants were transferred to a solid sucrose-supplemented MS medium containing 1mg.$l^{-1}$ BAP for six weeks. The micro-shoots derived from the multiplication stage were then immersed in a liquid sucrose supplemented MS medium containing antimitotic substances for 48–hrs, rinsed and then transferred onto a solid sucrose supplemented MS medium. At this stage a large number of micro-shoots of both *D. bellidiformis* and *M. whiteii* were lost to contamination (Tables 3.3 and 3.4). The persistence of contamination suggests that the surface sterilization procedure used to sterilize the seeds was inefficient at removing endogenous contaminants. More effective sterilization procedures should be implemented in the future to remove these contaminants.

The relatively high humidity in culture tubes has also been shown to promote contamination of explants cultured *in vitro*. Initially the treated micro-shoots were transferred into culture tubes that were closed with a cap and then sealed with Parafilm to increase the humidity in the culture vessel as described by Hannweg and Vos (2001, *pera comm.*). A large number of both *D. bellidiformis* (Table 3.3) and *M. whiteii* (Table 3.4) micro-shoots also showed hyperhydricity,phenolic exudation and necrosis. In an attempt to protect plant tissue cultures from infection and dessication (Jackson *et al.*, 1991), poor aeration often accompanies this preventative measure (Jackson *et al.*, 1991;

Zobayed *et al.*, 1999; Fal *et al.*, 2002 and Zobayed *et al.*, 2002). Sealed culture vessels facilitate the accumulation of ethylene and inhibits growth (Zobayed *et al.*, 1999). Therefore attempts to reduce this effect on the treated micro-shoots, the ventilation within the culture vessels was increased by not sealing the caps to the culture tubes with Parafilm. Although micro-shoots of *D. bellidiformis* showed no sign of necrosis, phenolic exudation and hyperhydricity (Table 3.5), the micro-shoots that did not show elongation were lost to contamination (Table 3.5). This also further suggests that the surface sterilization procedure applied was inefficient at removing endogenous contaminants in the seeds of *D. bellidiformis*.

Various studies have shown that hyperhydricity is a problem that effects plant regeneration *in vitro* (Letouze and Daguin, 1987; Rugini *et al.*, 1987; Jones *et al.*, 1993 and Kevers *et al.*, 2004). According to Kevers *et al.* (2004), hyperhydricity can be defined as a stress-induced change in physiological state which results from growth and culture conditions and stress factors under *in vitro* culture conditions that include: wounding and infiltration with soft culture medium, generally of a high ionic strength, rich in nitrogen and growth regulators in a special balance. In addition the explant is confined to a humid and gaseous environment which further contributes to stress. Pan and van Staden (1998) highlighted the use of activated charcoal in *in vitro* plant propagation to reduce the effect of culture conditions on numerous stress responses including phenolic exudation and hyperhydricity. McCartan and Crouch (1998) showed that hyperhydricity was reduced in *Mondia whiteii* micro-shoots that were cultured in the presence of 0.2% activated charcoal after a 4 to 6 week culture period. The addition of activated charcoal to a culture medium was also shown to reduce phenolic exudation from the explant (van Waes, 1987 and Torres 1989). In the current investigation the addition of 0.2% activated charcoal was only successful in eliminating the effect of hyperhydricity in *M. whiteii* micro-shoots cultured for four weeks on a solid sucrose-supplemented MS medium. The incorporation of activated charcoal in the *D. bellidiformis* medium was an ineffective protocol as the explants were lost to contamination or became necrotic. Hartman *et al.* (1997) and Torres (1989) suggest that in order to prevent the accumulation of gaseous and phenolic compounds,

reducing the time between subcultures is important. Therefore *M. whiteii* micro-shoots were transferred onto sucrose-supplemented MS medium containg 0.2% activated charcoal for four weeks (Table 3.8). While this eliminated necrosis, it also reduced hypehydricity and phenolic exudation.

Necrosis that was observed in both species could have been the effect of the 48 hr treatment with antimitotic substances based on the results from ultratructural studies on meristematic cells of shoot tips from seedlings of *D. bellidiformis* germinated in their presence. Another factor that could also be considered important in the response of the micro-shoots was the transfer of the explants from a liquid medium with a pH of 7 to a solid medium with a pH of 5.7. The shift in pH could have had a negative effect on the growth response of the micro-shoots. Although previous investigations have reported on the efficiency of immersing plant material in a liquid solution of an antimitotic substance to induce polyploidy (Lyrene and Perry, 1982), in the current investigation the method of inducing polyploids by immersing the plant material into a liquid medium with a high sucrose content containing an antimitotic substance was inefficient in producing a large number of polyploid individuals, the immersion method needs to be re-investigated. It is also evident from the different responses of the two species to the *in vitro* polyploidization conditions, that different species respond differently to the same polyplidization treatment.

## 4.4 DETERMINING POLYPLOID STATUS OF TREATED EXPLANTS

Flow cytometry was used to determine the ploidy status of treated *M. whiteii* micro-shoots. A number of reports have shown the efficiency of flow cytometry to determine ploidy status of plant cells (Eeckhuant *et al.*, 2004; Hannweg, 2004; Dolezel, 1997; Cohen and Yao; 1996; Awoleya *et al.*, 1996). In the present study the ploidy status was only determined for leaf samples of *M. whiteii* micro-shoots that were treated with antimitotic substances. From the flow cytometry analysis it was evident that changes in ploidy level occurred in micro-shoots that were initially treated with 0.01% oryzalin, while mixaploids were identified in micro-shoots initially treated with 0.1% colchicine and 0.01%

oryzalin. While van Tuyl *et al.* (1992) showed that successful regeneration and polyploidization was efficiently carried out in *Nerine* and *Lilium in vitro*, only 10% polyploidy was achieved in *M. whiteii* micro-shoots treated with 0.01% oryzalin and 0.001% oryzalin was not successful in inducing polyploidy in *M. whiteii* shoots.

Hannweg and Vos (2001, *pers comm.*) showed that 0.01% colchicine treatment was efficient in inducing polyploidy in several indigenous plant species (Table 1.3). However, in the current investigation, 10% of the *M. whiteii* micro-shoots treated were mixaploids following treatment with 0.01% colchicine. In the present study, micro-shoots derived from nodal cuttings from *in vitro* germinated seedlings were treated with antimitotic substances. Hannweg and Vos (2001, *pers comm.*) treated *M. whiteii* with colchicine by immersing nodal cuttings of *in vitro* germinated seedlings to induce polyploidy, while Cohen and Yao (1996) showed that successful polyploids of *Zanthedeschia* could be obtained from immersing micro-shoots into a solution of antimitotic substances. While the use of micro-shoots for *in vitro* polyploidization was successful for *Zanthedeschia* (Cohen and Yao, 1996) it was not successful in the current investigations with *D. bellidiformis* and *M. whiteii*, showing a species-specific response.

While Dolezel and Binarvo (1989) and Geoffriau *et al.* (1997) were able to successfully induce polyploidy with a 24-48hr immersion in a solution of antimitotic substance, in the current investigation only a limited number of plantlets were polyploid following a 48hr treatment with colchicine and oryzalin. Therefore there is scope for further investigations on the efficiency of polyploidy based on the response of plant material to antimitotic substances over time.

# CONCLUSION AND FUTURE RECOMMENDATION

In the present study the initial surface sterilization treatment tested was inefficient in removing endogenous contaminants, since contamination persisted during later culture. Therefore it is necessary to consider more efficient sterilization procedures than the one used in the present investigation. The impact of antimitotic substances on germination was determined. The ultrastructural response of *D. bellidiformis* to antimitotic treatments was evaluated. It was evident that plant cells respond differently to oryzalin and colchicine even at the same concentrations.

This study also aimed at inducing polyploidy individuals from *M. whiteii* and *D. bellidiformis* micro-shoots, however the practical constraints that limit *in vitro* plant tissue culture including contamination, exudation of phenolics, necrosis and hyperhydricty were found to limit the regeneration of treated micro-shoots and future studies to eliminate or reduce this problem is important. Mixaploid, triploid and diploid individuals were easily identified using flow cytometry and small leaf samples of treated individuals. Polyploidy individuals were only obtained from micro-shoots of *M. whiteii*, these individuals are currently being hardened-off and field trials will be set up with these individuals to identify superior genotypes.

While *in vitro* polyploidization techniques have offered a much easier method of manipulating plant chromosome number, several constraints associated with *in vitro* plant tissue culture should be considered in order to achieve high survival rate of 'mutated' individuals.

# REFERENCES


ABBOT, A.J. and ATKIN, R.K. 1987. Improving vegetatively propagated crops. Academic Press Limited, London. Pp 1 – 2.

ALLEMANN, J., LAURIE, S.M., THIART, S. and VORTSER H.J. 2004. Sustainable production of root and tuber crops (potato, sweet potato, indigenous potato, cassava) in southern Africa. South African Journal of Botany 70: 60 – 66.

APPLETON, M.R. and van STADEN, J. 1995. Micropropagation of some South African *hypoxis* plant species with medicinal and horticultural potential. Acta Horticulturae 420: 75 - 77.

APPLETON, M.R. and van STADEN, J. 1995. *In vitro* propagation of miniature hypoxis, *H. angustifolia*. Acta Horticulturae 420: 95 - 97.

ARTVINILI, S. 1987. Cytoskeletons, microtubules, tubulin and colchicine: a review. Cytologia 52: 189 – 198.

ASHLEY, T. and WAGENAAR, E. B.1974. Telomeric associations of gametic and somatic chromosomes in diploid and autotetraploid *Ornithogalum virens*. Canadian Journal of Genetics and Cytology 19: 61 – 76.

AUSTIN, R.B., MORGAN, C.L., FORD, M.A and BHAGWAT, S.G. 1982. Flag leaf photosynthesis of *Triticum aestivuum* and related tetraploid and diploid species. Annals of Botany 49: 177 – 189.

AWOLEYE, F., van DUREN, M., DOLEZEL, J. and NOVAK, F.J. 1996. Nuclear DNA content and *in vitro* induced somatic polyploidization in cassava (*Manihot esculata* Crantz) breeding. Euphytica 76: 196 – 202.

AVIVI, L. and FELDMAN, M, 1980. Arrangement of chromosomes in the interphase nucleus of plants. Human Genetics 55: 281 – 295.

BARRET, H.C. 1974. Colchicine induced polyploidy in citrus. Botanical Gazette 135: 29 – 41.

BARTISH, I.V., KORKHOVOV, V.I., FOMINA, Y.L. and LIM, Y.K. 1998.A new approach to obtain polyploid forms of apple. Acta Horticulturae 484: 561 – 564.

BECK, S.L. 2003. Evaluation of induced polyploidy in *Acacia mearnsii* through stomatal counts and guard cell measurements. South African Journal of Botany 69: 563 – 567.

BENNETT, M.D. 1972. Nuclear DNA content and minimum generation time in herbaceous plants. Procceedings of the Royal Society of London 181: 109 – 135.

BLEAZARD, W. McCAFFERY, J. M., KING, E. J., BALE, S. MOZDY, A., TIEU, Q., NUNNARI, J., SHAW, J. M. 1999. The dynamin-related GTPase Dnm1 regulates mitochondrial fission in yeast. Nature Cell Biology 1: 298 – 304.

BORZON, Z., ZOLDOS, V., KRSTINIC, A. and PAPES, D. 1994. Ploidy of some arborescent willow clones in relation to their production in field tests. In: Borzon, Z. and Schlarbaum, S. E. (eds): Cytogenetic studies of forest trees and shrub species.

BROERTJES, C and HARTEN, A.M. 1988. Applied Mutational Breeding for Vegetatively Propagated Crop. Elsevier, Amsterdam. pp 3 – 15; 25 – 28.

BURDON, J. J. and MARSHALL, D. R. 1981. Inter-and-intra-specific diversity in the disease response of *Glycine* species to the leaf-rust fungus *Phakopsora phachyrhizi.* Journal of Ecology 69: 381 – 390.

BURGOYNE, P.M. 2004. Molecular advances in the family *Mesembryanthaceae*: Implications for our current few of mesembs. South African Association of Botanists, Annual Congress, Durban. p 50.

BYRNE, M. C., NELSON, C. J. and RANDALL, D. D. 1981. Ploidy effects on anatomy and gas exchange of tall fescue. Plant Physiology 68: 891 – 893.

CHAUVIN, J. E., SOUCHET, C., DANTEC, J.P. and ELLISSÉCHE, D. 2003. Plant Cell, Tissue and Organ Culture 73: 65 – 73.

CHAVDEJ, S. and BECKER, H. 1984. Influence of colchicine treatment on Chromosome number and growth rate of tissue cultures of *Valeriana wallichi* DC. Plant Cell, Tissue and Organ Culture 3: 265 – 272.

CHUKWUJEKWU, J.C., FENNELL, C.W., van STADEN, J. 2002. Optimisation of a tissue culture protocol for the endangered *Aloe poyphylla*. South African Journal of Botany 68: 424 – 429.

COETZEE, C., JEFTHAS, E. and REINTEN, E. 1999. Indigenous plant genetic Resources. In: J. Janick (ed), Perspectives on new crops and new uses. ASHS press, Alexandra, VA. Pp 160 – 163.

COHEN, D. and YAO, J. 1996. *In vitro* chromosome doubling of nine *Zanthedeschia* cultivars. Plant Cell, Tissue and Organ Culture 47: 43 – 49.

COMAI, L. 2000. Genetic and epigenetic interactions in allopolyploid plants. Plant Molecular Biology. 43: 387 – 399.

COOK, E.L., CUNNINGHAM, A. and van STADEN, J. 1988. The tissue culture of an exploited Zulu medicinal plant, *Bowiea volubilis*. South African Journal of Botany 54: 509 – 510.

CULLIS, C. and DAVIES, D. R. 1994. Ribosomal RNA cistron number in a polyploidy series of plants. Chromosoma 46: 23 – 28.

CUNNINGHAM, T. 1989. Over-exploitation of medicinal plants in Natal/KwaZulu: Root Causes. Veld and Flora: 85 – 87.

CUSHMAN, J.C., WULAN, T., KUSCUOGLU, N., SPARTZ, M.D. 2000. Efficient plant regeneration of *Mesembryanthemum crystallinum via* somatic embryogenesis. Plant Cell Report 19: 459 – 463.

DAWE, R. K. 1998. Meiotic chromosome organisation and segregation in plants. Annual Review of Plant Physiology and Molecular Biology 49: 371 – 395.

DeBERGH, P., AITKEN-CHRISTIE, J., COHEN, D., GROUT, B., von ARNOLD, S., ZIMMERMAN, R. and ZIV, M. 1992. Reconsideration of the term "vitrification" as used in micropropagation. Plant Cell, Tissue and Organ Culture 30: 135 – 140.

De LANGE, H., TENANT, S., BOTHA, P., KLEIN, C. and NICHOLS, G. 1989. Micropropagation and the trade in indigenous medicinal plants. Veld and Flora: 60 – 61.

DeMAINE, M.J. and SIMPSON, G. 1999. Somatic chromosome number doubling of selected potato genotypes using callus culture or the colchicine treatment of shoot nodes *in vitro*. Annals of Applied Biology 134: 125 – 130.

DeMARIE, E.T. 1998. Southern African Plants in North American greenhouses-unrealized Potential. Veld and Flora: 17 – 19.

DIRO, M. and van STADEN, J. 2003. *In vitro* regeneration of *Ensete ventricosum* from zygotic embryos of stored seeds. South African Journal of Botany 69: 364 – 369.

DOLEZEL, J., BINAROVA, P. and LUCRETTI, S. 1989. Analysis of nuclear DNA content in plant cells by flow cytometry. Biologia Planta 31: 113 – 120.

DOLEZEL, J., BINAROVA, P. and LUCRETTI, S. 1989. Analysis of nuclear DNA content in plant cells by flow cytometry. Biologia Plantarum 31: 113 – 120.

DOLEZEL, J. 1997. Flow cytometry, its application and potential for plant Breeding. In: LELLY, T. (ed). Current Topics in Plant Cytogenetics Related to Plant improvement. WUV-Universitatsverlag, Australia. Pp 80 - 90.

ECKARDT, A.N. 2001. A sense of self: The role of DNA sequence elimination in allopolyploidization. Plant Cell 13: 1699 – 1704.

EECKHAUNT, T. SAMYN, G. and van BOCKSTAELE, E. 2002. *In vitro* polyploidy induction in *Rhododendron simsii* hybrids. Acta Horticulturae 572: 43 – 49.

EECKHAUNT, T. G., WERBROUCK, S. P., LEUS, L. W., van BOCKSTAELE, E. J. and DEBERGH, P.C. 2004. Chemically induced polyploidization in *Spathiphyllum wallisii* Regel through somatic embryogenesis. Plant Cell, Tissue and Organ Culture 78: 241 – 246.

ENGELMANN, F.1997. *In vitro* Conservation Methods. In: CALLOW, J.A, FORD-LLOYD, B.V. and NEWBURY, H.J. (eds). Biotechnology and Plant Genetic Resources: Conservation and Use: CAB International, New York. pp 119 – 159.

ERIKSON, H. P. 2000. Dynamin and FtsZ: missing links in mitochondrial and bacterial division. The Journal of Cell Biology 148: 1103 – 1106.

FAL, M.A., MAJADA, J.P and SANCHEZ TAMES, R. 2002. Physical environment In non-ventilated culture vessels effects *in vitro* growth and morphogenesis of several cultivars of *Dianthus caryophyllus* L. *In vitro* Cellular and Development Biology – Plant 38: 589 – 596.

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

FENNELL, C.W. and van STADEN, J. 2004.Biotechnology of southern African bulbs. South African Journal of Botany 70: 37 -46.

FRASER, L.G. HARVEY, C.F. and KENT, J. 1992. Ploidy manipulation of kiwifruit in tissue culture. Acta Horticulturae 297: 109 – 114.

FUSSEL, C. P. 1975. The position of interphase chromosomes and late replicating DNA in centromere and telomere regions of *Allium cepa* L. Chromosoma 50: 201 – 210.

GAMBORG, O. L. and SHYLUK, J. P. 1981. Nutrition, media and characteristics of plant cell and tissue cultures. In: T. A. Thorpe (ed), Plant tissue culture: Methods and Applications in Agriculture, pp 21 – 44. Academic Press.

GAO, S.L., ZHU, D.N., CAI, Z.H. and XU, D.R. 1996. Autotetraploid plants from colchicine- treated bud culture of *Salvia miltiorrhiza* Bge. Plant Cell, Tissue and Organ Culture 47: 73 – 77.

GAO, M., DAVIS, D. and BIRCHLER, J. A. 1996. Dosage effects on gene expression in a maize ploidy series. Genetics 142: 1349 – 1355.

GEOFFRIAU, E. KAHANE, R. BELLAMY, C. and RANCILLAC, M. 1997. Ploidy stability and *in vitro* chromosome doubling in gynogenic clones of onion (*Allium cepa* L.). Plant Science 122: 201 – 208.

GEORGE, E.F. 1993. Plant Propagation by Tissue Culture. P1 2nd Edition. Exgetics Limited, Edington, Wilts, England. Pp 3-14, 37 – 63.

GRACE, O.M., JAGER, A.K., van STADEN, J. 2003. Bark medicines used in traditional healthcare in KwaZulu-Natal, South Africa: An inventory. South African Journal of Botany 69: 301 – 363.

GRIESBACH, R.J. 1985. Polyploidy in *Phaelaenopsis* orchid improvement. Journal of Heredity 76: 74 – 75.

GRIESBACH, R.J. and BHAT, R.N. 1990. Colchicine-induced polyploidy in *Eustoma grandiflorum*. Hort-Science 25: 1284-1286.

GUNNING B. E. and HARDHAM, A. R. 1982. Microtubules. Annual Review of plant Physiology 33: 651 – 698.

HA, S., MOORE, P.H., HEINZ, D., KATO, S., OHMIDO, N. and FAKUI, K. 1999. Quantitative chromosome map of the polyploid *Saccharum spontaneum* by multicolor fluorescence *in situ* hybridization and imaging methods. Plant Molecular Biology 39: 1165 – 1173.

HANCOCK, J.F., 1997. The colchicine story. Hort-Science 32: 1011 - 1012.

HANNWEG, K. WATT, M.P. and BERJAK, P. 1996. A simple method for the micropropagation of *Bowiea volubilis* from inflorescence explants. Botanical Bulletin of Academia Sinica 37: 213 – 218.

HANNWEG, K. F. and VOS, J.E. 2001. The Agricultural Reasearch Council – Institute for Tropical and Subtropical Crop, Nelspruit.

HANNWEG, K. F. 2002. The Agricultural Reasearch Council – Institute for Tropical and Subtropical Crop, Nelspruit.

HANNWEG, K.F. 2004. Enhancing desirable characteristics in indigenous plants through the induction of polyploidy. South African Association of Botanists, Annual Congress, Durban . Pp23.

HARTMAN, H.T., DALE, E.K., DAVIES, F.T. and GENEVE, R.L.1997. Plant Propagation – Principles and Practices. 6th Edition. Prentice Hall, Upper Saddle River, New Jersey. Pp 549 – 611.

HASSAN, L., JONES, R.N., PARKER, J.S. and POSSELT, U.K. 1991. Colchicine induced heritable variation in cell size and chloroplast number in the leaf cells of inbred ryegrasses (*Lolium perenne, L. multiflorum*). Euphytica 52: 39 – 45.

HASSAWI, D. S. and LIANG, G.H. 1991. Antimitotic agents: Effects on Double haploid production in wheat. Crop Science 31: 723 – 726.

HEINZ, D.J. and MEE G.W. P. 1970. Colchicine induced polyploids from cell suspension cultures of sugarcane. Crop Science 10: 696 – 699.

HILU, K.W. 1993. Polyploidy and the evolution of domesticated plants. American Journal of Botany 80: 1494 – 1499.

HINSHAW, J. E. and SCHMID, S. L. 1995. Dyanimin self-assembles into rings suggesting a mechanism for coated vesicle budding. Nature 374: 190 – 192.

HOL, G.M. and van der LINDE, P. 1992. Reduction of contamination in bulb explant cultures of *Narcissus* by a hot water treatment of parent bulbs. Plant Cell, Tissue and Organ Culture 31: 75 – 79.

HUSBAND, B.C. 1999. Constraints on polyploid evolution: a test of the minority cytotype exclusion principle. Proceedings of the Royal Society of London 267: 217 – 223.

INGLE, J., TIMMIS, J. N. and GORE, J. R. 1976. In: N. Sunderland (ed), Percpectives in Experimental Biology 2, pp 273 – 281. Pergoman, New York.

JACKSON, M.B., ABBOT, A.J., BELCHER, A.R., HALL, K.C., BUTLER, R. and CAMERON, J. 1991. Ventilation in plant tissue cultures and effects of poor aeration on ethylene and carbon dioxide accumulation, oxygen depletion and explant development. Annals of Botany 67: 229 – 237.

JIANG, C., WRIGHT, R. J., EL-ZIK, K. M. and PATERSON, A. H. 1998. Polyploid formation created unique avenues for response to selection in *Gossypium*. Proceedings of the National Academy of Sciences 95: 4419 – 4424.

JONES, N.B., DRENNAN, P.M. and van STADEN, J. 1993. Leaf anatomy, chloroplast organization and photosythetic rate of hyperhydric *Eucalyptus Saligina* Sm material. South African Journal of Botany 59: 651 – 655.

KATO, M. 1989. Polyploids of Camellia through culture of somatic embryos. Hort-Science 24: 1023 – 1025.

KEVERS, C., FRANCK, T., STRASSER, R.J., DOMMES, J. and GASPAR, T. 2004. Hyperhydricity of micropropagated shoots: a typically stress-induced change of physiological state. Plant Cell, Tissue and Organ Culture 77: 181 – 191.

KUNNEMAN, B.P. and FAAIJ-GROENEN, G.P. 1988. Elimination of bacterial Contaminants: a matter of detection and transplanting procedures. Acta Horticulturae 225: 183 – 188.

KOORBANALLY, N. A., MULHOLLAND, D.C. and CROUCH, N. R. 2000. Isolation of Isovanillin from aromatic roots of the medicinal African liane, *Mondia whiteii*. Journal of Herbs, Spices and Medicinal plants 7: 37 – 43.

KOWALSKI, B. and van STADEN, J. 2001. Micropropagation of *Podocarpus henkelii* and *P. elongates*. South African Journal of Botany 67: 362 – 366.

LEE, H. S. and CHEN, Z.J. 2001. Protein coding genes are epigenetically regulated in *Arabidopsis* polyploids. Proceedings of the National Academy of Sciences 96: 6753 – 6758.

LEIFERT, C. and CASSELLS, A.C. 2001. Microbial hazards in plant tissue and cell cultures. *In Vitro* Cellular and Developmental Biology - Plants 37: 133 – 138.

LEIFERT, C. and WAITES, W.M. 1990. Contaminants of plant tissue cultures. International Applied Plant Tissue Culture Newsletter 60: 2 – 5.

LETOUZÉ, R and DAGUIN, F. 1987. Control of vitrification and hypolignification process in *Salix babylonica* cultured *in vitro*. Acta Horticulturae 212: 185 – 192.

LEVIN, D. A. 1983. Polyploidy and novelty in flowering plants. American Naturalist 122: 1 – 25.

LINDSEY, K. and JONES, M.G.K. 1989.Plant Biotechnology in Agriculture. Biddles Limited, Great Britian, Guildford and Kings Lynn, pp 1-14; 57 – 76.

LI, W., BERLYN. G.P. and ASHTON, P.M. 1996. Polyploids and their structural and physiological characteristics relative to water deficit in *Betula papyrifera* (Betulaceae). American Journal of Botany 83: 15 – 20.

LYRENE, P. M. and PERRY, T.L. 1982. Production and selection of blueberry polyploids *in vitro*. Journal of Heredity 73: 377 – 378.

MAJADA, J.P., TADEO, F., FAL, M.A. and SANCHES-TAMES, R. 2000. Impact of culture vessel ventilation on the anatomy and morphology of micropropagated carnation. Plant Cell, Tissue and Organ Culture 63: 207 – 218.

MANDER, M. 1999. Indigenous medicine (muthi) trade. (Available online at http:// www.ceroi.net/reports/durban/Terrestri/muthi.htm)

MARTNINEZ-PEREZ, E., SHAW, P.J. and MOORE, G. 2000. Polyploidy induces centromere association. The Journal of Cell Biology 148: 233 – 238.

McALISTER, B.G., JAGER, A.K. and van STADEN, J. 1998. Micropropagation of *Babiana* spp. South African Journal of Botany 64: 88 – 90.

McCARTAN, S.A. and CROUCH, N.R. 1998. *In vitro* culture of *Mondia whiteii* (Periplocaceae) A threatened Zulu medicinal plant. South African Journal of Botany 64: 313 – 314.

McCARTAN, S.A. and van STADEN, J. 2002. Micropropagation of *Scilla Kraussii* and *Scilla dracomontana.* South African Journal of Botany 68: 223 – 225.

McCARTAN, S.A. and van STADEN, J. 1999. Micropropagation of members of the Hyacinthaceae with medicinal and ornamental potential – a review. South African Journal of Botany 65: 361 – 369.

McNEVIN, M. A., PITTS, H. C. and YOON, Y. 2000. The dynamin family of mechanoenzymes: pinching in new places. Trends in Biochemical Sciences 25: 115 – 120.

MOREJOHN, L. C. and FOSKET, D. E. 1984. Taxol-induced rose microtubule polymerization *in vitro* and its inhibition by colchicine. Journal of Cell Biology. 99: 141 – 147.

MOREJOHN, L. C., BUREAU, T. E., BAJER, A. S., FOSKET, D. E. 1987. Oryzalin, a dinitroaniline herbicide, binds to plant tubulin and inhibits microtubule polymerization *in vitro*. Planta 172: 252 – 264.

MURASHIGE, T. and SKOOG, F. 1962. A revised medium for rapid growth and bio assays with tobacco tissue cultures. Physiologia Planta 15: 473 – 497.

MURASHIGE, T. 1977. Plant cell and organ cultures as horticultural practices. Acta Horticulturae 78: 17 -29.

NELSON, A. D. an ELISON, W.J. 1999. Polyploid evolution and biogeography in chelone (Scrophulariaceae): Morphological and isozyme evidence. American Journal of Botany 86: 1487 – 1501.

NEUBAUER, J. and THOMAS, H. L. 1966. The effects of various colchicine pH levels of seed treatment on polyploid cells and other cytological variations in root tips of red clover. Crop Science 6: 209 – 210.

NIEDERWIESER, J.G. and VCELER, B.M. 1990. Regeneration of *Lachenalia* species from leaf explants. Hort-Science 25: 684 – 687.

NIEDERWIESER. J.G. 2004. Role of Biotechnology in the development and production of *Lachenalia* and *Ornithogalum* cultivars in South Africa. South African Journal of Botany 70: 47 – 51.

NIEDZ, R.P. and BAUSHER, M.G. 2002. Control of *in vitro* contamination of explants from greenhouse and field-grown trees. *In vitro* Cellular and Developmental Biology - Plant 38: 468 – 471.

NIGRO, S.A., MAKUNGA, N.P., GRACE, O.M. 2004. Medicinal plants at the etnobotany-biotechnology interface in Africa. South African Journal of Botany 70: 89 –96.

NORTH, C. 1976 Artificial chromosome doubling in Narcissus and its implication for breeding N. Tazetta hybrids. Acta Horticulturae 63: 161 - 164.

OKOLE, B.N. 2004. Commercialisation of plants in Africa. South African Journal of Botany 70: 109 -115

OSTERYOUNG, K. W. 2000. Organelle fission. Crossing the evolutionary divide. Plant Physiology 123: 1213 – 1216.

OTTO, S.P. and WHITTON, J. 2000. Polyploid incidence and evolution. Annual Review of Genetics 34: 401 – 437.

OZKAN, H., LEVY, H.H. AND FELDMAN, M. 2001. Allopolyploidy-induced rapid genome evolution in the wheat (Aegilops-Triticum) group. Plant Cell 13: 1735 – 1747.

PAN, M.J and van STADEN, J. 1998. The use of Charcoal in *in vitro* culture – A review. Plant Growth Regulation 26: 155 – 163.

PAPPE, L.1847. A List of South African Indigenous Plants, used as Remedies by Colonists of the Cape of Good Hope. G.J. Pike Publishers, Cape Town, South Africa. Pp 6 - 7.

PÂQUES, M. and BOXUS, P. 1987. "Vitrification": review of literature. Acta Horticulturae 212: 155 – 166.

PETERSON, K.K., HAGBERG, P., KRISTIANSEN, K. 2003. Colchicine and oryzalin mediated chromosome doubling in different genotypes of *Miscanthus sinensis*. Plant Cell, Tissue and Organ Culture 73: 137 – 146.

PICKETT-HEAPS, J.D. 1974. Plant Microtubules. In: ROBARDS, A.W. (ed). Dynamic aspects of Plant Ultrastructure. McGraw-Hill Book Company Limeted, UK.

PREDIERI, S., MALAVASI, F. F. F. and FILITI, N. 1989. *In vitro* colchicine treatment on strawberry (*Fragaria* X *Ananassa Duch*) shoots. Acta Horticulturae 265:

PRYOR, R.L. and FRANZIER, L.C. 1968. Colchicine induced tetraploid azaleas. Hort-science 3: 283 - 286.

RATHNAM, C.K.M. and CHOLLET, R. 1980. Photosynthetic and photorespiratory carbon metabolism in mesophyll protoplasts and chloroplasts isolated from isogenic diploid and tetraploid cultivars of ryegrass (*Lolium perenne L.*). Plant Physiology 65: 489 – 494.

REED, B.M. and TANPRASERT, P. 1995. Detection and control of bacterial contaminants of plant tissue cultures. A review of recent literature. Plant Tissue Culture and Biotechnology 1: 137 –142.

REYNOLDS, E. S. 1963. The use of lead citrate at high pH as an electron hopaque stain in electron microscopy. Journal of Cell Biology 17: 206 – 212.

ROMERO-ARANDA, R. BONDADA, B. R., SYVERSTSEN, J.P. and GROSSER, J. W. 1997. Leaf characteristics and net gas exchange of diploid and autotetraploid citrus. Annals of Botany 79: 153 – 160.

RUGINI, E., TARINI, P., ROSSODIVITA, M.E., COSTANZO, S. 1987. Control of shoot "vitrification" of almond and olive grown *in vitro*. Acta Horticulturae 212: 177 – 184.

SANDERS, H. and HULL, J. W. 1970. Dimethyl sulfoxide as an adjuvant of colchicine in the treatment of *Rubus* seeds and shoot apices. Hort-Science 5: 111 – 112.

SHAO, J., CHEN, C., DENG, X. 2003. *In vitro* induction of tetraploid Pomegranate (*Punica granatum*). Plant Cell, Tissue and Organ Culture 75: 241 – 246.

SCHAPENDONK, A.H.C.M., SPITTERS, C.J.T. and De VOS, A.L.F. 1990. Comparison of nitrogen utilization of diploid and tetraploid perennial ryegrass genotypes using a hydroponic system. In: Bassen N.E.R. (eds), Genetic aspects of plant mineral nutrition., pp 299 – 306.

SCHEID, O.M., JAKOVLEVA, L., AFSAR, K., MALUSZYNSKA, J. and PASZKOWSKI, J. 1996. A change of ploidy can modify epigenetic silencing. Proceedings of the National academy of Sciences 93: 7114 - 7119.

SCHIFINO, M.T. and MOREAS-FERNANDES, I. M. 1987. Induction of polyploidy and cytological characterization of autotetraploids of *Trifolium riograndense* Burkart (leguminosae). Euphytica 36: 863 – 872.

SHUSHU, D.D. 2001. *In vitro* regeneration of the Kalahari devil's claw, *Harpagophytum procumbens*, an important medicinal plant. South African Journal of Botany 67: 378 – 380.

SIMMONDS, N.W. and SMARTT, J. 1999. Principles of Crop Improvements. 1st Edition. Blackwell Science Limited, Oxford, London. Pp 262 – 283.

SIVALOPOV, A. L., and BLAGODARVO, T.A. 1994. Different levels of mixoploidy in hybrid poplars. In: Borzan, Z. and Schlarbaum, S.E. (eds): Cytogenic studies of forest trees and shrub species pp 311 – 316.

SONG, K. and OSBORN, T. C. 1994. A method of examining homologus genes in plant polyploids. Plant Molecular Biology 26: 1065 – 1071.

SONG, K.; LU, P.; TANG, K. and OSBORN, T.C. 1995. Rapid genome change In synthetic polyploids of *Brassica* and its implications for polyploid evolution. Proceedings of the National Academy of Science 92: 7719 – 7723.

SOLTIS, D.E. and SOLTIS, P. S. 1995. The dynamic nature of polyploidy genomes. Proceedings of the National Academy of Science 92: 8089 – 8091.

SOLTIS, D.E. and SOLTIS, P.S. 1999. Polyploidy: recurrent formation and genome evolution. Trends in Ecology and Evolution 14: 348 – 352.

SOLTIS, P.S. and SOLTIS, D.E. 2000. The Role of genetic and genomic attributes in the success of polyploids. Proceedings of the National Academy of Sciences 97: 7051 – 7057.

SOOST, R.K. 1987. Breeding Citrus – Genetics and Nucellar Embryony. In: ABBOT, A.J. and ATKIN, R.K. (eds). Improving Vegetatively Propagated Crops. Academic Press Limited, London. Pp 83 – 110.

SPURR, A. R. 1969. A low viscosity epoxy resin embedding medium for electron Microscopy. Journal of Ultrastructure Research 26: 31 – 43.

TAIRA, T., SHAO, Z.Z., HAMAWAKI, H. and LARTER, E.N. 1991. The effect of colchicine as a chromosome doubling agent for wheat-rye hybrids as influenced by pH, method of application and post-treatment environment. Plant Breeding 106: 329 – 333.

TAKAMURA, T. and MIYAJIMA, I. 1996. Colchicine induced tetraploids in yellow-Flowered cyclamens and their characteristics. Scienta Horticulturae 65: 305 – 308.

TAL, M. 1977. Physiology of polyploid plants: DNA, RNA, protein and abscisic acid in autotetraploid and diploid tomato under low and high salinity. Botanical Gazzette 138: 119 – 122.

TAL, M. and GARDI, I. 1976. Physiology of polyploid plants: water balance in autotetraploid and diploid tomato under low and high salinity. Physiology Plantarum 38: 257 – 261.

TALUKDAR, K. and SEN, S. 1997. Intraspecific genetic diversity in *Allium ascalonicum*. Acta Horticulturae 433:

TETYANA, P. and van STADEN, J. 2001. Micropropagation of *Cussonia paniculata* – a medicinal plant with horticultural potential. South African Journal of Botany 118: 367 – 370.

THAO, N.T.P., URESHINO, K., MIYAJIMA, I., OZAKI, Y. and OKUBO, H. 2003. Induction of tetraploids in ornamental *Alocasia* through colchicine and oryzalin treatments. Plant Cell, Tissue and Organ culture 72: 19 – 25.

THOMAS, P. 2004. *In vitro* decline in plant cultures: detection of a legion of covert bacteria as the cause of degeneration of long-term micropropagated triploid watermelon cultures. Plant Cell, Tissue and Organ Culture 77: 173 – 179.

THOMPSON, J.N. CUNNINGHAM, B.M., SEGRAVES, K.A., AALTOFF, D.M. and WAGNER, D. 1997. Plant polyploidy and insect/plant interactions. American Naturalist 150: 730 – 743.

TORRES, K.C. 1989. Tissue Culture Techniques for Horticultural Crops. Van Nostrand, Reinhold, New York. pp 1-66.

VÄINÖLÄ, A.N.U. 2000. Polyploidization and early screening of Rhododendron hybrids. Euphytica 112: 239 – 244.

VÄINÖLÄ, A.N.U. and REPO T. 2001. Polyploidisation of *Rhododendron* cultivars *in vitro* and how it effects cold hardiness. Acta Horticulturae 5560: 319 – 322.

van JAARSVELD, E.1996. Veld Gardening in South Africa. Veld and Flora: 52 – 53.

van RENSBURG, W.S.J. 2004. Role of indigenous leafy vegetables in combating hunger and malnutrition. South African Journal of Botany 70: 52 – 59.

van STADEN, J. 1998. Biotechnology of medicinal plants-case studies from South Africa. Acta Horticulturae 461: 87 – 428.

van TUYL, J. M., De VRIES, J. N., BINO, R. J. and KWAKKENBOS, A. A. M. 1989. Identification of 2n-pollen producing interspecific hybrids of *Lilium* using flow cytometry. Cytologia 54: 737 – 745.

van TUYL, J.M., MEIJER, B. and van DIEN. 1992. The use of oryzalin as an alternative for colchicine in *in vitro* chromosome doubling of *Lilium* and *Nerine*. Acta Horticulturae 325: 625 – 630.

van WAES, I. 1987. Effect of activated charcoal on *in vitro* propagation of Western European orchids. Acta Hoticulturae 212: 131 – 138.

van WYK, B.E. 2002. A review of ethnobotanical research in Southern Africa. South African Journal of Botany 68: 1 – 13.

VEGA, J.M. and FELDMAN, M. 1998. Effect of the Pairing Gene *Ph1* on Centromere Misdivision in Common Wheat. Genetic 148: 1285 – 1294.

VERHOEVEN, H.A., SREE RAMULU, K. and DIJKHUIS, P. 1990. A comparison of the effects of various spindle toxins on metaphase arrest and formation of micronuclei in cell-suspension cultures of *Nicotiana plumbaginifolia*. Planta 182: 408 – 414.

VOLNEC, J. J. 1988. Herbage growth and carbohydrate metabolism of diploid and tetraploid alfalfa. Crop Science 28: 128 – 132.

WACHIRA, F. N. and NG'ETICH, W. K. 1999. Dry-matter production and partition in diploid, triploid and tetraploid tea. Journal of Horticultural Science and Biotechnology 74: 507 – 512.

WENDEL, J.F. 2000. Genome evolution in polyploids. Plant Molecular Biology 42: 225– 249.

WETHERELL, D.F. 1982. Introduction to *in vitro* propagation. Wayne,N.J., Avery Publishers.

WU, J. H. and MOONEY, P. 2002. Autotetraploid tangor plant regeneration from *in vitro* Citrus somatic embryogenic callus treated with colchicine. Plant Cell, Tissue and Organ Culture 70: 99 – 104.

ZOBAYED, S.M., AMSTRONG, J. and ARMSTRONG, W. 2002. Multiple shoot Induction and flower bud abscission of Annona cultures as affected by types of ventilation. Plant Cell, Tissue and Organ Culture 69: 155 – 165.

ZOBAYED, S.M., ARMSTRONG, J. and ARMSTRONG, W. 1999. Cauliflower shoot-culture: effects of different types of ventilation on growth and physiology. Plant Science 141: 209 – 217.

ZSCHOCKE, S. and van STADEN, J. 2000. *In vitro* propagation of *Tulbaghia simmleri*. South African Journal of Botany 66: 86 – 89.