INDUCTION OF POLYPLOIDY IN *EUCALYPTUS* SPECIES AND INTERSPECIFIC HYBRIDS

By

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PREFACE

The experimental work described in this dissertation was carried out in the School of Biochemistry, Genetics, Microbiology and Plant Pathology and also in the School of Biological and Conservation Sciences at the University of KwaZulu-Natal, under the supervision of Professor Richard Beckett and the co-supervision of Professor Annabel Fossey.

I hereby certify that this statement is correct, and as the candidate's Supervisors we agree/~~do not agree~~ to the submission of this dissertation.

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2008

DECLARATION

I **TRACY MARITZ** declare that

(i) The research reported in this dissertation, except where otherwise indicated, is my original work.

(ii) This dissertation has not been submitted for any degree or examination at any other university.

(iii) This dissertation does not contain other persons' data, pictures, graphs or other information, unless specifically acknowledged as being sourced from other researchers.

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TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

ABBREVIATIONS

AMs	Apical meristems
ANOVA	Analysis of variance
CSIR	Council for Scientific and Industrial Research
DF	Degrees of freedom
g	Grams
h	Hours
ICFR	Institute for Commercial Forestry Research
GLMM	Generalized linear mixed model
mg	Milligrams
ml	Millilitres
MS	Mean square
n	Sample size
PI	Propidium iodide
p value	p-probability statistic
PVP	polyvinylpyrrolidone
SAMs	Shoot apical meristems
SD	Standard deviation
SS	Sum of squares
REML	Restricted maximum likelihood
μl	Microlitres
μm	Micrometers
2n	Somatic number
2x	Diploid basic number
4x	Tetraploid basic number
20 ×	Twenty times (magnification)
40 ×	Forty times (magnification)
100 ×	One hundred times (magnification)

ABSTRACT

A large sector of the forestry industry of South Africa comprises *Eucalyptus* species, covering approximately 49% of the forestry plantation area. Polyploidy induction has become an attractive tool to increase yield and reduce invasiveness in forestry species. Polyploidy induction in *Eucalyptus* using colchicine treatments on seed and axillary buds was undertaken to produce tetraploids that could be used in breeding programmes; specifically to increase yield and decrease species invasiveness through the production of triploids after crossing with diploid parents.

Eight seedlots of *E. urophylla* and seven of *E. grandis* were treated with four colchicine concentrations (0.00, 0.01, 0.03, 0.05%) at two exposure times (18 h and 24 h), treating two seeds per treatment, repeated eight times. For axillary bud induction, 20 buds of two *E. grandis* clones and three *E. grandis* × *E. urophylla* hybrids and one *E. grandis* × *E. nitens* hybrid were treated with four colchicine concentrations (0.0, 0.5, 1.0, 1.5%) for three consecutive days. A known tetraploid hybrid *E. grandis* × *E. camaldulensis* and its corresponding diploid were included as reference material.

Seedlings and bud sports were pre-screened by determining stomatal guard cell lengths. Seedlings and bud sports displaying cell lengths significantly ($p<0.0001$) larger than the diploid were selected as putative polyploids. Polyploidy was then confirmed by quantifying the DNA content using flow cytometry. Stomatal frequencies and guard cell chloroplast frequencies were also determined in the induced tetraploid seedlings to evaluate their suitability to discern between ploids.

All putative polyploidy seedlings, identified in the pre-screening process, were confirmed, using flow cytometry, as either tetraploids or mixoploids. Of the 17 *E. urophylla* putative polyploids, from various seedlots, six were tetraploid and 11 mixoploid. In *E. grandis* one of the five putative polyploids, from various seedlots, was tetraploid and four mixoploid. Pre-screening of bud sports was less accurate; only four of the 12 *E. grandis* hybrid putative polyploids were mixoploid and only three of the six *E. grandis* putative polyploids were mixoploid.

E. urophylla seedlings were more sensitive to colchicine than *E. grandis* seedlings displaying a lower survival rate (52%) than *E. grandis* (63%). Extreme treatments that caused the lowest survival rates were also responsible for most of the polyploidy successful inductions; 0.05%/18 h and 0.05%/24 h for *E. urophylla* and 0.03%/24 h and 0.05%/24 h for *E. grandis.*

Phenotypic effects of colchicine included shorter, thicker roots and hypocotyls; darker leaves; longer and narrower leaves in some tetraploids; and asymmetrical leaf margins in many mixoploids and tetraploids compared with the controls. In the tetraploids, stomata were significantly larger ($p<0.0001$) and less frequent ($p<0.001$). A significant ($p<0.001$) increase in the number stomatal chloroplasts was also ascertained.

Confirmed mixoploid seedlings all displayed tetraploid leaves based on stomatal size and thus classified as periclinal chimeras. In bud sports, only leaves with islands of diploid and tetraploid stomata in the confirmed mixoploids were encountered. Mixoploid bud sports were thus either sectional or mericlinal chimeras.

Stomatal size proved to be a suitable pre-screening method, especially in polyploidy induction in seedlings. Additionally confirmed tetraploids exhibited significantly different stomatal frequencies and stomatal chloroplast frequencies compared with the diploids, thus proving to be suitable detection methods for polyploidy screenings. Polyploidy induction in seed was effective, however, less effective in axillary buds which requires further research to refine methods.

CHAPTER 1
INTRODUCTION

1.1 INTRODUCTION

Worldwide, natural and plantation forests cover approximately four billion hectares (ha) of land (FAO, 2001). Of this land 95% comprise natural forests, while the remaining 5% is assigned to plantation forests, approximately 200 million ha of land (FAO, 2001; Siry *et al.,* 2005) (Table 1.1).

Table 1.1 Area covered by natural and plantation forests on the different continents (adapted from FAO, 2001).

Region	**Total forest area** (000 ha)	**Natural forest area** (000 ha)	**Plantation forest area** (000 ha)
Africa	649 866	645 829	4 037
Asia	547 793	482 364	65 429
Oceania	197 623	194 775	2 848
Europe	1 039 251	943 160	96 091
North America	470 564	445 812	24 752
Central America	78 740	78 092	648
South America	885 618	875 163	10 455
World in total	3 869 455	3 665195	204 260

Natural forests play an important role in the livelihood of many people throughout the world providing shelter, firewood, furniture and many other forest related products (Palo and Uusivuori, 1999). They also play an important role in the preservation of ecosystems, providing shelter and food for a diverse range of organisms and also contribute to the maintenance of the atmospheric carbon balance (Siry *et al.*, 2005). Commercial forestry, on the other hand, plays a key role in the economy of many countries by firstly, providing employment (Tewari, 2001) and secondly, providing

timber for construction, infrastructure and for the production of paper and pulp as well as tannins for the leather tanning industry (Poynton, 1979).

Most commercial forests worldwide comprise predominately two genera, *Pinus* and *Eucalyptus* (FAO, 2001). In South Africa, both *Pinus* and *Eucalyptus* species contribute to approximately 90% of all commercial forests covering approximately 1.3 million ha (Department of Water Affairs and Forestry, 2005). The remainder of the commercial forests comprises mostly *Acacia* species (Department of Water Affairs and Forestry, 2005) (Figure 1.1).

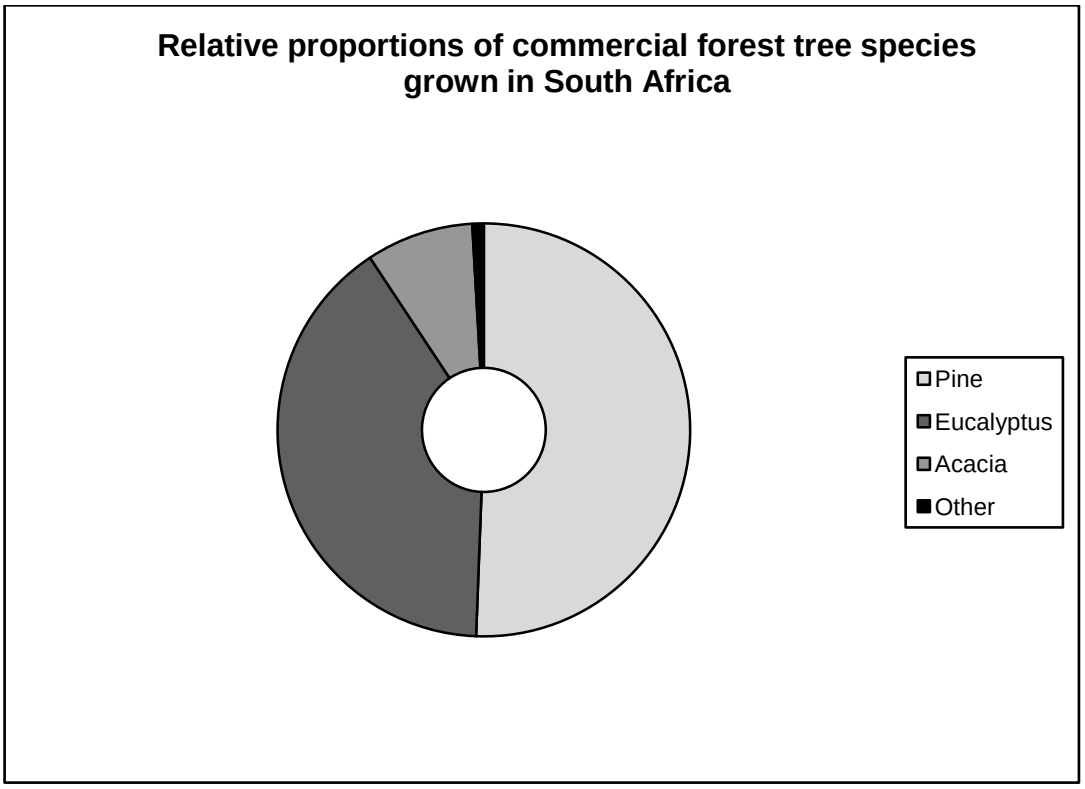

Figure 1.1 Relative proportions of commercial forest tree species grown in South Africa (adapted from Department of Water Affairs and Forestry, 2005).

1.2 *EUCALYPTUS*

The genus *Eucalyptus* is a flowering plant that belongs to the family Myrtaceae (Rye, 1979). *Eucalyptus* originates from the Australasia region, where approximately 700 species have been recognised (Delaporte *et al.,* 2001; Potts, 2004). Within the genus the chromosome compliment is relatively uniform (Eldridge *et al.*, 1993), where the majority of the species have a chromosome complement of 2n = 2x = 22, while a small proportion has 2n = 2x = 24 (Rye, 1979).

The different species of *Eucalyptus* are fast growing and highly adaptable and have colonised a diverse range of environmental conditions within their natural habitats (Poynton, 1979; Coppen, 2002). Intraspecific variation is high, forming provenances, populations within a species that have adapted to a variety of different environmental conditions (van Wyk and Verryn, 2000). It is through their highly tolerant nature of drought and pests (Eldridge *et al.*, 1993); coupled with the wide array of uses offered by many of the species (Potts, 2004) that has encouraged cultivation and proliferation in foreign countries (Eldridge *et al.*, 1993; Potts, 2004).

1.3 *EUCALYPTUS* FORESTRY IN SOUTH AFRICA

1.3.1 Introduction

South Africa was one of the first countries in the southern hemisphere to introduce *Eucalyptus* species during the early 19th century (Owen and van der Zel, 2000). South Africa is fairly poor in natural forest resources (Tewari, 2001), and therefore plantations comprising of *E. globulus*, *E. camaldulenesis* and *E. tereticornis* were established during the late 1800's (Penfold and Willis, 1961; Owen and van der Zel, 2000). Today, *Eucalyptus* plantations comprise 40.1%, 531 849 ha of the 1.3 million ha of commercial forested South African land (Department of Water Affairs and Forestry, 2005). Of the land covered by *Eucalyptus* plantation forests, *E. grandis* is the most economically important species covering 295 876 ha of land, 55% of the total land planted to *Eucalyptus* plantations (Department of Water Affairs and Forestry, 2005). The remaining 45% comprises other *Eucalyptus* species and

Eucalyptus species hybrids (Department of Water Affairs and Forestry, 2005). *Eucalyptus* is utilized in a wide range of products such as construction and building material, transmission poles, mining supports, furniture wood, as well as pulp and paper production (Owen and van der Zel, 2000).

Eucalyptus plantations are found mostly in the provinces of KwaZulu-Natal and Mpumalanga in South Africa (Department of Water Affairs and Forestry, 2008). The temperate regions consist of mostly cold tolerant species such as *E. dunnii*, *E. macarthurii*, *E. nitens* and *E. smithii* (Swain and Gardner, 2004), while the warmer sub-tropical regions consist predominantly of *E. grandis* and *E. grandis* hybrids (Department of Water Affairs and Forestry, 2008).

1.3.2. Breeding and improvement

Traditionally, *Eucalyptus* domestication has evolved through the application of conventional breeding strategies (Potts, 2004). In conventional breeding, repeated cycles of breeding and selection are performed on initial populations, intraspecific and interspecific hybridization undertaken and superior genotypes selected for production (van Wyk and Verryn, 2000). However, in recent times the application of biotechnology has also contributed to the domestication of this species (FAO, 2001) (Figure 1.2). Various biotechnological methods that are implemented in *Eucalyptus* tree breeding include transformation (Potts, 2004) and polyploidy induction (Kampoor and Sharma, 1984).

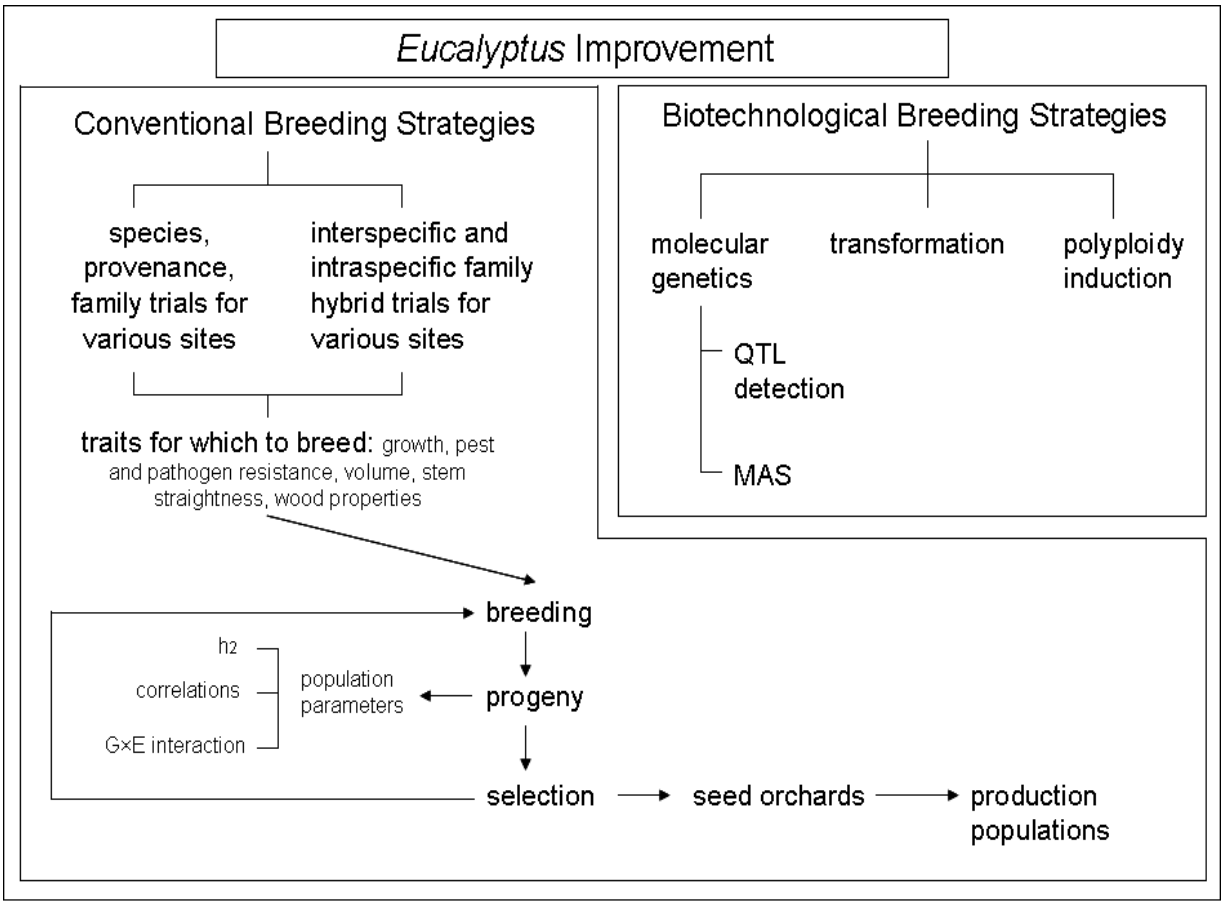

Figure 1.2 Breeding strategies in *Eucalyptus.*

Conventional breeding strategies

The cytologically stable nature of *Eucalyptus* has played a major role in its breeding and has allowed for the combination of traits between a wide range of different species through interspecific hybridisation, between species within the genus, and intraspecific hybridisation, between provenances within particular species (Zobel and Talbert, 1984).

Breeding strategies address a variety of different traits. Economically important wood quality traits include, wood density, fibre length, stem straightness, growth rate and log-end splitting (Eldridge *et al.*, 1993). Other desirable traits that are also included in breeding strategies are pathogen and pest resistance, as well as drought and cold tolerance (Eldridge *et al.*, 1993).

The major components of conventional breeding involve selection, progeny testing and crossing, which form the framework of breeding cycles (van Wyk and Verryn, 2000). A breeding cycle is initiated by the establishment of a base population, which serves as a resource of genetic diversity from which breeding populations and production populations are developed (van Wyk and Verryn, 2000). Successive cycles may include progeny testing, which permit the evaluation of important genetic parameters that are central to the selection process (Fins *et al.*, 1992). These parameters include the determination of variance components which are used to calculate individual breeding values, heritabilities for certain traits in a population for a particular site, and correlations that are useful in calculating genotype by environmental (G × E) interaction (Figure 1.3) (Fins *et al.*, 1992).

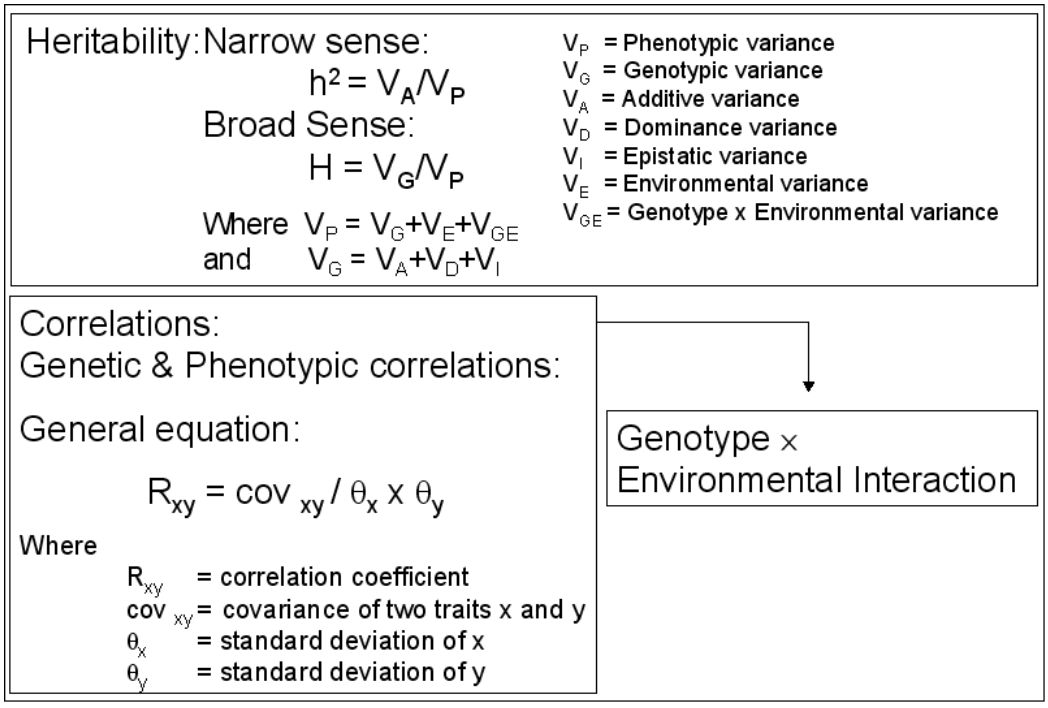

Figure 1.3 Equations used to calculate population parameters for breeding purposes.

Breeding strategies are generally varied, complex, multi-faceted and specific to particular breeding programmes (Fins *et al.*, 1992). Depending on the breeding objectives, trees are either open pollinated or control pollinated (Wright, 1976). Open pollination allows for trees to receive pollen from a wide range of different trees

establishing comprehensive genetic combinations, while controlled pollination is more precise involving the crossing of two pre-selected genotypes (Wright, 1976).

Various selection procedures can also be implemented into breeding strategies depending on what trait or traits the breeder is selecting (Fins *et al.*, 1992). These procedures include tandem selection, independent culling and index selection (Stonecypher, 1970). Tandem selection is the consecutive selection of desired traits (Poehlman and Sleper, 1995) and is classified as single trait selection (Stonecypher, 1970). Single trait selection is rare in forestry breeding, owing to the long rotation period hence greater genetic gains are achieved when multiple trait selection is implemented (Stonecypher, 1970). Independent culling involves scoring each individual for each trait; individuals are then selected when all traits meet certain scoring criteria (Poehlman and Sleper, 1995). The use of selection indices have proven to be successful in selection of superior individuals in multiple trait selection, where each individual is ranked taking into account certain parameters of the desired traits such as heritability and phenotypic variance (Stonecypher, 1970).

The chromosome compliment that exists between different *Eucalyptus* species allows for the combination of diverse genotypes through interspecific hybridization (Zobel and Talbert, 1984). Interspecific hybridization has become a prominent breeding strategy in *Eucalyptus* breeding (van Wyk and Verryn, 2000; Potts, 2004), utilising hybrid vigour and creating novel gene combinations (Wright, 1976). In South Africa many of the hybrids have been constituted with *E. grandis* as one of the parent species, for example, the desirable hybrids of *E. grandis* × *E. urophylla* show fast growth from *E. grandis* and increased disease resistance and coppicing potential of *E. urophylla* (Eldridge *et al.*, 1993). Additionally, a host of hybrids have been produced to improve economically important traits and to broaden the site planting range of *Eucalyptus* (Potts and Dungey, 2004).

Biotechnological strategies

Conventional breeding is typically a slow process in long rotational crops and may take decades before improvement in a particular trait is noted (Fossey, 2005). Often, more precise methods are practiced congruently to conventional breeding programmes to aid in the improvement of a particular species (van Wyk and Verryn, 2000). A number of technologies have in recent times gained prominence in tree breeding, such as the use of transformation (Potts, 2004) and the use of molecular markers in selection (Marker assisted selection, MAS) (FAO, 2001; Potts, 2004).

Although many genetically modified varieties have been produced through transformation in agriculture (Fossey, 2005), this type of genetic improvement has been relatively sluggish in *Eucalyptus* (Potts, 2004). Transgenic *Eucalyptus* species such as *E. grandis*, *E. saligna* and *E. urophylla* have been tested in field trials in a number of countries such as South Africa, Spain and United Kingdom (Potts, 2004). However, it will take over a decade until genetically modified clones are planted on a large scale, which, in turn, has resulted in increased interest in molecular breeding (Potts, 2004).

Molecular breeding has proven to be a valuable tool in improving the effectiveness of breeding programmes in agriculture (Varshney *et al.*, 2005) as well as in forestry (Grattapaglia, 2004). DNA fingerprinting uses molecular markers to create physical maps to characterise genomes providing knowledge of certain genomic regions associated with phenotypic variation of quantitative traits; named quantitative trait loci (QTL) (Varshney *et al.*, 2005). In *Eucalyptus*, QTLs for growth, density and stem straightness have been identified and used in MAS (Grattapaglia, 2004; Potts, 2004). This technology further facilitates early screening of genotypes, where conventional breeding would require phenotypic screening at an older age when the desirable trait has manifested (Butcher and Southerton, 2007).

In addition to molecular techniques, polyploidisation has been used for the production of novel phenotypes for a number of purposes (Eeckhaut *et al.*, 2004). It is usually expected that chromosome doubling leads to larger cells and subsequently greater yields (Elliot, 1958). In *Eucalyptus*, polyploidy has been induced in *E. citriodora* to

investigate its effects on the yield of citronellal and citronellol oils (Janaki *et al.,* 1969). Furthermore, polyploidy has been induced in a hybrid of *E. grandis* and *E. tereticornis* by Kampoor and Sharma (1984) to investigate its potential to increase timber yield. However, the performance of artificial polyploids in hardwood species in the long term is largely unknown as indicated by the dearth of available literature.

1.4 POLYPLOIDY

1.4.1 Introduction

Most plants and animals are diploid and possess two sets of chromosomes (Kehr, 1996). Polyploidisation, the multiplication of the number of chromosome sets, is a natural phenomenon that occurs relatively frequently in many plant taxa, but rarely in animal taxa (Thompson and Lumaret, 1992). Polyploids contain more than two sets of chromosomes (>2x) in their somatic cells (2n) (Kehr, 1996) and are usually classified according to the number of basic chromosome sets (x); for example, triploid (3x), tetraploid (4x), pentaploid (5x), etc. (Darlington, 1973).

Generally an increase in the number of chromosome sets is accompanied by substantial changes in the phenotype, physiology and fertility of the polyploid (Levin, 1983). Manifestations of these changes include enlarged vegetative parts, such as larger flowers (Eeckhaut *et al.*, 2004), fruit and roots (Elliot, 1958). The characteristics associated with chromosome doubling in plants are often common to many polyploids of different species (Dermen, 1940).

The multiple sets of chromosomes in polyploids often lead to complications with synapsis of chromosomes during meiosis (Darlington, 1973). This results in the formation of multivalents and monovalents, which tend to lead to unequal segregation of chromosomes and consequently the production of unbalanced gametes and resultant infertility and male sterility (Darlington, 1973). Infertility in many polyploids manifests as seedless fruits (Elliot, 1958). A summary of the prominent characteristics of polyploids is provided in Table 1.2.

Table 1.2 Characteristics of polyploids.

Characteristic	Description in polyploidy	Reference
Enlarged cells	The cells of polyploids (including epidermal and stomatal cells) are larger than those of diploids to compensate for the increase in genetic material. The organelles are also typically larger such as the nuclei, chloroplasts and mitochondria.	Stebbins (1950) and Levin (1983).
Increased cell volume	The increase in cell size consequently causes an increase in cell volume.	Levin (1983) and Kehr (1996).
Heightened susceptibility to frost damage	The increased cell volume causes an increase in the water content of cells, which consequently renders some species susceptible to frost damage.	Vainola and Repo (2001).
Reduced growth rate	The reduced number of cell divisions due to the increase in genetic material causes a decrease of the growth rate.	Frost (1925) and Wright (1962).
Increased organ size	The increase in cell size consequently results in certain organs that are abnormally large. However, the increase in size is not always correlated to the degree of ploidy.	Elliot (1958), Romero-aranda *et al.* (1997) and Vainola and Repo (2001).
Late flowering	Both the period until flower formation begins and flowering itself are prolonged. This is caused by a generally reduced growth rate and decreased rates of metabolism.	Elliot (1958) and Levin (1983).

Decreased fertility	A decreased fertility is generally associated with autopolyploidy and segmental allopolyploidy. The variable meiosis of these polyploids results in unbalanced gametes and consequently a decreased fertility.	Stebbins (1950) and Darlington (1973).
Increased fertility	Some allopolyploids may display an increase in fertility. This depends upon the genome combination before polyploidisation. If the genomes occur in duplicate, the gametes will be balanced and the individual fertile.	Stebbins (1950), Rieseberg (2001), Eeckhaut *et al.* (2004) and Nimura *et al.* (2006).
Higher resistance to pests and pathogens	Polyploidy is often associated with a change in physiology whereby there is an increase or decrease in certain metabolites within the plant. Consequently, polyploids may have an increase in metabolites active against pests and pathogens, conferring an increased resistance.	Levin (1983) and Eeckhaut *et al.* (2004).
Increased heterozygosity	The increase in chromosome number results in an increase in the number of alleles per locus and consequently more alleles in a state of heterozygosity. This may confer an increased adaptability on the plant.	Levy and Feldman (2002), Soltis and Soltis (2000) and Khosravi *et al.* (2008).
Asexual reproduction	Polyploidy is often associated with asexual reproduction. Especially autopolyploids that are known for their infertility have evolved to reproduce asexually.	Stebbins (1950), Thompson and Lumaret (1992) and Levy and Feldman (2002).

The altered characteristics of polyploids are attractive alternatives for plant breeders (Elliot, 1958). For example, the forestry industry is interested in polyploids because of the potential to increase volume and to reduce fertility to potentially curb prolific seed production of invasive species such as black wattle (Mathura *et al.*, 2006).

Artificial polyploid induction began early in the 20th century (Elliot, 1958). Induction methods included subjecting germline material of seed and meristematic tissue to chemicals and temperature extremes (Blakeslee and Avery, 1937), which permitted breeders to create an array of varieties not available in their diploid relatives (Kehr, 1996).

1.4.2 Natural polyploidy and classification

The incidence of naturally occurring polyploids in plants varies between genera and families (Otto and Whitton, 2000). In flowering plant species (the angiosperms) the occurrence of polyploidy, averages at approximately 50% (Soltis and Soltis, 2000), while in the pteridophytes the incidence of polyploidy is estimated to average 95% (Soltis and Soltis, 2000; Levy and Feldman, 2002).

Natural polyploids are mostly formed through either somatic chromosome doubling or by the fusion of unreduced gametes during sexual reproduction (Bretagnolle and Thompson, 1995; Ramsey and Schemske, 1998; Otto and Whitton, 2000). Polyploidy that arises asexually in meristematic cell(s) occurs through somatic chromosome doubling when somatic (non-reproductive) mutations occur, due to a disruption in mitosis (Otto and Whitton, 2000). A polyploid shoot results in the formation of a chimeric plant, comprising of more than one cell line (Ramsey and Schemske, 1998). The most frequent route of polyploid formation is through gamete non-reduction (Bretagnolle and Thompson, 1995); failure of normal segregation of chromosomes during microsporogenesis or megasporogenesis, resulting in unreduced diploid gametes (Bretagnolle and Thompson, 1995; Ramsey and Schemske, 1998).

Polyploids are generally classified according to the types of chromosome sets (genomes) present and how the polyploids could have originated (Darlington, 1973).

In instances where all the genomes have the same karyotype the polyploid could have originated through somatic means or through an intraspecific cross (Singh, 2002). These polyploids are referred to as autopolyploids (Singh, 2002; Osborn, 2004). On the other hand, when polyploidy results from interspecific crosses, where the genomes have different karyotypes, they are known as allopolyploids (Singh, 2002; Osborn, 2004).

Autopolyploids contain multiples of the same genome (Soltis and Soltis, 2000); for example a tetraploid will have four genomes with the same karyotype denoted with the same letter of the alphabet such as AAAA or BBBB. The chromosomes of these polyploids are homologous and therefore display polysomic inheritance and irregular meioses with multivalents and monovalents, which results in varying degrees of infertility (Otto, 2007). In nature these polyploids circumvent infertility through asexual reproduction (Levin, 1983).

Allopolyploids, also referred to as amphidiploids, are the products of hybridisation between different species (Thompson and Lumaret, 1992). The different genomes with different karyotypes (Elliot, 1958; Soltis and Soltis, 2000) are denoted by different letters of the alphabet such as AABB for an allotetrapolyploid. Allopolyploids vary in the degree of fertility (Soltis and Soltis, 2000). In instances where the genomes are distantly related and the chromosomes of the different genomes do not synapse during meiosis, fertility could be high, up to 100% (Otto, 2007). In contrast, when the homoeologous chromosomes of the different genomes tend to synapse during meiosis, irregular meioses results and semi-fertility arises to varying degrees (Singh, 2002). Irregular meiosis is due to heterozygosity between structural rearrangements of the different genomes; such as inversions, duplications, deletions and translocations (Soltis and Soltis, 2000). These allopolyploids are referred to as segmental allopolyploids (Elliot, 1958) and their genomes described using the same letter of the alphabet but differentiated with super- or subscripts; for example for the segmental allotetrapolyploid the genomes may be written as $A^1A^1A^2A^2$. Figure 1.4 provides a flow diagram of the formation routes and descriptions of different tetrapolyploids.

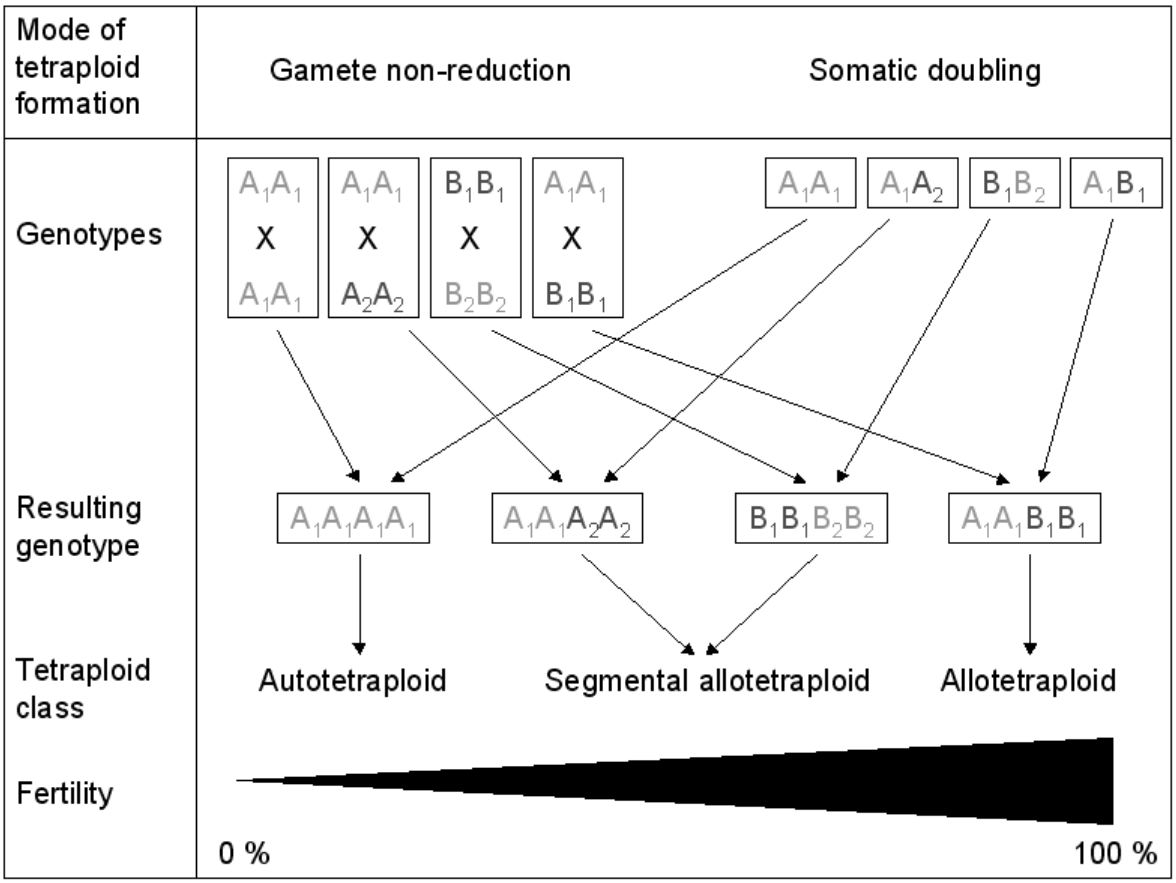

Figure 1.4 Outline of the formation routes and descriptions of different tetrapolyploids.

1.5 POLYPLOIDY INDUCTION

In an effort to produce crops with the desirability of natural polyploids, plant breeders are able to select from a variety of methods to induce polyploidy (Elliott, 1958). These methods include temperature alterations (Randolph, 1932) biotechnological methods, such as the manipulation of protoplast and callus cultures, (Slater *et al.,* 2003) and chemicals (Blakeslee and Avery, 1937).

1.5.1 Temperature induction of polyploidy

The use of temperature was one of the earliest methods employed to induce polyploidy (Dermen, 1940). Cold shock treatments and heat treatments have been successfully used to induce polyploidy (Randolph, 1932; Dermen, 1940) in a few

species: maize (Randolph, 1932) corn, barley and wheat (Dermen, 1940). Due to the unreliable results obtained from temperature treatments, the use of other more reliable methods were suggested by Blakeslee and Avery as early as in 1937.

1.5.2 Biotechnological induction of polyploidy

The fusion of protoplasts has also been employed to create polyploids (Slater *et al.,* 2003). Protoplasts are produced by the removal of cell walls either mechanically or enzymatically (Chawla, 2002). However, enzymatic removal of cell walls is the preferred method as the yield of protoplasts is greater than using mechanical means (Slater *et al.,* 2003). Protoplasts are fused either through the application of chemicals or through electrofusion (Bravo and Evans, 1985) and has been successfully used to induce polyploidy in *Nicotiana tabacum* and *Datura innoxia* as well as in chicory (Rambaud *et al.*, 1992).

Callus cultures, masses of undifferentiated cells, tend to exhibit a range of cell variants that include altered gene expression, polyploidy and aneuploidy (Phillips *et al.,* 1994; Sangthong *et al.*, 2005). The expression of polyploidy in somaclonal cell lines has been used to successfully isolate polyploids cells in Citrus (Slade Lee, 1988) and *Lilium longiflorum* (Sangthong *et al.*, 2005).

1.5.3 Chemical induction of polyploidy

Colchicine, an alkaloid originating from *Colchicum autumnale* (Lehrer *et al.*, 2008), was first used to induce polyploidy in *Datura* (Blakeslee and Avery, 1937), and later in other plants such as *Acacia mearnsii* (Moffett and Nixon, 1960), *Trifolium riograndense* (Schifino *et al.,* 1987) and *Colophospermum mopane* (Rubuluza *et al.,* 2007). The success of this alkaloid lies in its ability to prevent the formation of microtubules during cell division, mitosis (Dhooghe *et al.*, 2009). Consequently, the chromosomes lapse into daughter chromosomes and reorganise into a single nucleus; now with the number of chromosome sets doubled (Eeckhaut *et al.*, 2002).

In recent years the use of more accessible, cheaper (Zlesak *et al.*, 2005) and less toxic chemicals than colchicine have been investigated (Hansen and Andersen,

1996; van Duren *et al.*, 1996). These include oryzalin (van Duren *et al*, 1996), trifluralin (Eeckhaut *et al.*, 2002) and nitrous oxide (Taylor *et al.*, 1976).

Oryzalin, a dinitroaniline herbicide (Hugdahl and Morejohn, 1993) has the ability, like colchicine, to prevent microtubule formation (Morejohn *et al.*, 1987). The chemical attaches to tubulin, preventing polymerisation of the microtubules, thereby preventing further lengthening of the microtubules (Morejohn *et al.*, 1987) and subsequently disrupts the normal segregation of chromosomes (Hugdahl and Morejohn, 1993). The chromosomes then lapse into daughter chromosomes resulting in chromosome doubling (Eeckhaut *et al.*, 2002). Oryzalin has been found to be more effective at lower concentrations than colchicine (van Tuyl *et al.*, 1992; van Duren *et al.*, 1996) in *Rhododendron simsii* (Eeckhaut *et al.*, 2002), *Alocasia* (Thao *et al.,* 2003) and *Spathiphyllum wallisii* Regel (Eeckhaut *et al.*, 2004)

Trifluralin, like oryzalin, is a dinitroaniline herbicide (Eeckhaut *et al.*, 2004) and displays a similar action to oryzalin against microtubules (Eeckhaut *et al.*, 2002). Trifluralin has also been shown to be more effective than colchicine in *R. simsii* hybrids (Eeckhaut *et al.*, 2002), *S. wallisii* Regal (Eeckhaut *et al.*, 2004) and *Rosa chinensis minima* (Sims) (Zlesak *et al.*, 2005).

Another alternative to colchicine is nitrous oxide (N_2O), which has been successfully used to induce polyploidy in red clover (Taylor *et al.*, 1976). In red clover the number of chimeras (mixed cell types) was significantly less than the number found using colchicine (Taylor *et al.*, 1976).

Figure 1.5 provides a flow diagram of the most frequently used methods to induce polyploidy.

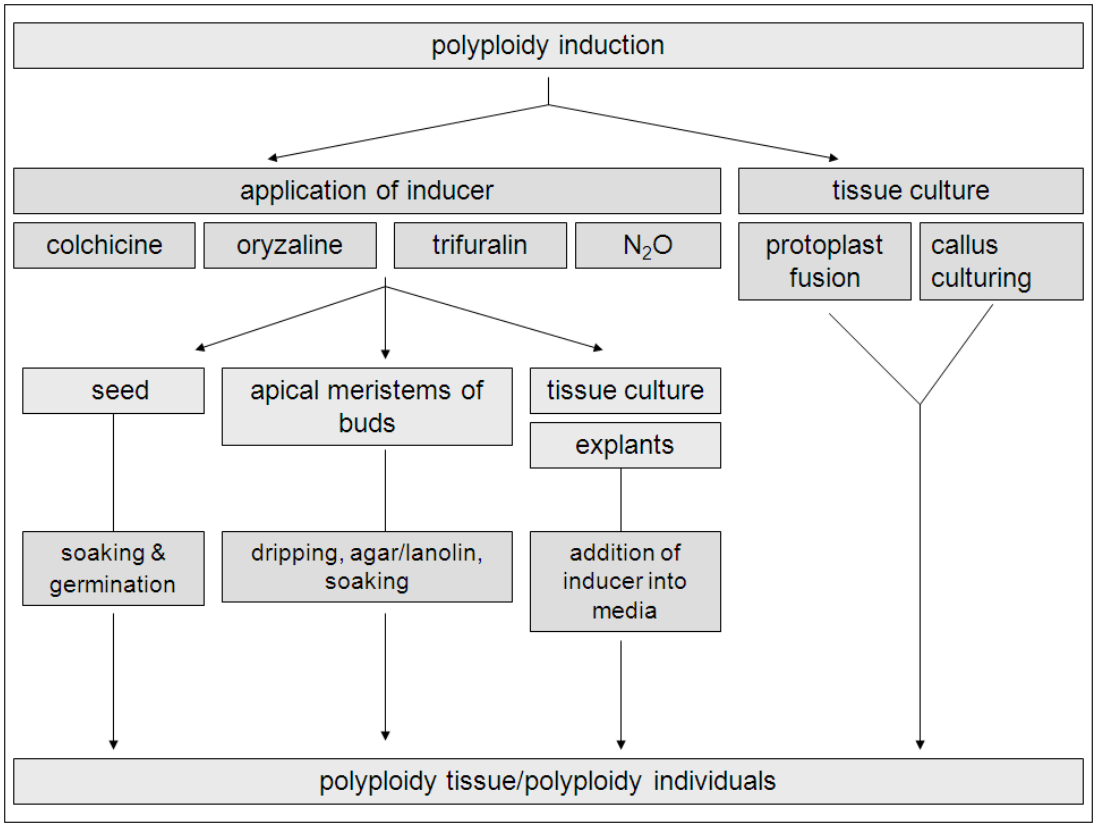

Figure 1.5 Most frequently used methods to induce polyploidy.

1.5.4 Plant tissues utilized in the induction of polyploidy

In polyploidy induction, chemicals are generally applied to actively dividing cells (Elliot, 1958). These include seeds, apical meristematic structures and actively dividing cells in culture (Dermen, 1940).

Seed

Seed is frequently used in chemical polyploid induction because of its high success rate (Dermen, 1940). The chemical inducers are usually applied to seed in two different ways: either by first soaking the seed in a solution of the chemical and then allowing the seed to germinate (Dermen, 1940; Avery *et al.*, 1947); or by germinating the seed directly in a solution of the chemical, usually on chemical-soaked filter paper, allowing the seed to imbibe the inducer (Harbard, personal communication, 2006; Ahokas, 1998; Rubuluza *et al.*, 2007). For successful induction of polyploidy, different concentrations of a particular chemical inducer

needs to be tested so as to employ the most effective concentration (Dermen, 1940). Prior soaking in colchicine before germination has been successfully used to induce polyploidy in *Datura* (Blakeslee and Avery, 1937) and in *Acacia mearnsii* (Beck *et al.*, 2003b). Similarly, oyzalin has been used to induce polyploidy in *Acacia dealbata* and *Acacia mangium* by Blakesley *et al.* (2002). Harbard (personal communication, 2006) has also successfully induced polyploidy in *A. mangium* by germinating seed in a solution of colchicine.

Apical meristems (AMs)

Shoot apical meristems (SAMs) are also used as a source of plant material in chemical polyploid induction (Dermen, 1940). These regions contain actively dividing cells (Dermen, 1940). SAMs used in polyploid induction are obtained from seedlings at the cotyledon stage (Zlesak *et al.*, 2005), or slightly older terminal buds of seedlings (Hofmeyr and van Elden, 1942), as well as axillary buds from older plants. The chemical inducer is applied to the SAMs by either dripping an aqueous solution of the chemical into the apical meristem (Blakeslee and Avery, 1937), or by mixing the chemical in lanolin or agar then applying the paste to the SAMs (Avery *et al.*, 1947), or lastly, by soaking the SAMs in an aqueous solution of the chemical inducer (Avery *et al.,* 1947). Dripping of aqueous chemical onto SAMs has been successfully employed in *Exacum* (Semeniuk, 1978), in *Trifolium riograndense* (Schifino and Fernandes, 1987) and in Rhododendrons (Eeckhaut *et al.*, 2002), and in *Rosa chinensis minima* (Zlesak *et al.*, 2005). Chemical containing lanolin or agar paste has also been used to induce polyploidy in *Carica papaya* (Hofmeyr and van Elden, 1942) and *Solanum* (Chauvin *et al.*, 2003). Soaking of SAMs has also been used to induce polyploidy in *T. riograndense* (Schifino and Fernandes, 1987).

Cells in tissue culture are another source of plant material used in polyploid induction (Shao *et al.,* 2003 and Yang *et al.,* 2006). The chemical inducer is usually added to the tissue culture medium (Shao *et al.*, 2003). This *in vitro* method of polyploidy induction has reported to yield more tetraploids and fewer chimeras than treating seed and axillary buds *ex vitro* (Shao *et al.*, 2003 and Yang *et al.,* 2006). Polyploidy has been successfully induced using colchicines supplemented media for the culturing of shoot tips of *Alocasia* (Thao *et al.*, 2003) and root tips of *Musa*

acuminata (van Duren *et al.*, 1996). Similarly oryzalin was used to induce polyploidy in scales from *Lilium longiforum* (Takamura *et al.*, 2002) and from *Nerine* (van Tuyl *et al.*, 1992).

Tables 1.3, 1.4 and 1.5 provide summaries of examples of plant material, application times and species used for the induction of polyploidy by colchicine, oryzalin and trifluralin respectively.

Table 1.3 Examples of polyploid induction using colchicine.

Species	Plant material	Concentration	Application	Time	Reference
Rosa chinensis minima	Cotyledons of seedlings	0.5%	5 µl of inducer suspended on SAMs	24 h	Zlesak *et al.* (2005)
Musa acuminata	SAM cultures	0.05, 0.25, 0.50, 0.75, 1.0%	Inducer in tissue culture medium	0.5, 2, 4 h	Hamill *et al.* (1992)
Carica papaya	Terminal buds of young seedlings	0.06 to 0.50%	Drop of inducer suspended on SAMs	2-3 times/day for 1 - 4 days	Hofmeyr and van Elden (1942)
Rhododendron	SAMs at cotyledon stage	0.25%	Drop of inducer suspended on SAMs	Twice daily for 7-10 days	Kehr (1996)
Alocasia	SAMs	0.01, 0.05, 0.10%	Inducer in tissue culture medium	24, 48 and 72 h	Thao *et al.* (2003)
Musa acuminata	Root tips	2.5, 5.0 and 10.0 mM	Inducer in tissue culture medium	48 h	Van Duren *et al.* (1996)
Lilium and *Nerine*	Scales	0.10%	Inducer in tissue culture medium	4 h	Tuyl *et al.* (1992)
Rhododendron	SAMs	0.025 and 0.050%	Inducer in tissue culture medium	24 and 48 h	Vainola and Repo (2001)

Table 1.4 Examples of polyploid induction using oryzalin.

Species	Plant material	Concentration	Application	Time	Reference
Lilium longiforum	Scales	0.001, 0.003, 0.005%	Soaking in inducer and tissue cultured	3 h	Takamura *et al.* (2002)
Alocasia	SAMs	0.005, 0.010, 0.050%	Inducer in tissue culture medium	24, 48 and 72 h	Thao *et al.* (2003)
Musa acuminata	Root tips	15, 30 and 60 µM	Inducer in tissue culture medium	7 days	Van Duren *et al.* (1996)
Lilium and Nerine	Scales	0.001 - 0.010%	Inducer in tissue culture medium	4 h	Van Tuyl *et al.* (1992)
Rhododendron	SAMs	0.025 and 0.050%	Inducer in tissue culture medium	24 and 48 h	Vainola and Repo (2001)
Rhododendron	SAMs at cotyledon stage	0.01 and 0.05%	Drop of inducer suspended on SAMs	Daily for 3 - 7 days	Eeckhaut *et al.* (2002)
Cassava	Axillary SAMs	15, 30 and 60 µM	Inducer in tissue culture medium	48 h	Awoleye *et al.* (1994)
Solanum	SAMs	2.88, 14.4 and 28.8 µM	Inducer in tissue culture medium	3 weeks	Chauvin *et al.* (2003)

Table 1.5 Examples of polyploid induction using trifluralin.

Species	Plant material	Concentration	Application	Time	Reference
Rosa chinensis minima	SAMs at cotyledon stage	0.0086 and 0.086%	5 µl of inducer suspended on SAMs	24 h	Zlesak *et al.* (2005)
Rhododendron	SAMs at cotyledon stage	0.01 and 0.05%	Drop of inducer suspended on SAMs	Daily for 3 – 7 days	Eeckhaut *et al.* (2002)
Brassica napus	Anthers	0.3, 1.0, 3.0, 10.0 and 30.0 µM	Inducer in tissue culture medium	12 h	Hansen and Andersen (1996)
Spathiphyllum wallisii	Anthers	1.0, 3.0, 10.0 and 30.0 µM	Inducer in tissue culture medium	6 weeks	Eeckhaut *et al.* (2004)

1.5.5 Results of induction of polyploidy

During the induction of polyploidy multiple layers of cells are exposed to a chemical polyploidy inducer (Dermen, 1940; Satina *et al.*, 1940). When the different layers of apical meristems are exposed to the chemical, it may happen that only some of the cells undergo polyploidisation, which consequently leads to an outgrowth from an induced apical meristem bud (bud sport) and a seedling from induced seed that contains two cell lines, each with a different number of chromosomes; the original cell line (diploid) and the newly formed polyploid cell line (Dermen, 1940).

Chimeras are plants that contain more than one cell line and are in essence mosaics (Szymkowiak and Sussex, 1996; Schepper *et al.,* 2001). They are defined as individuals that possess more than one genotype that grow parallel to one another in plant tissues (Nozawa and Hirata, 2002). Chimeras thus result when a change (mutation) occurs in the actively dividing cells of an apical meristem located either in buds or in seed (Szymkowiak and Sussex, 1996). The term cytochimera is used to describe chimeras that possess histogenic layers that vary in ploidy (Burge *et al.*, 2002).

Formation of chimeras in apical meristems (AMs)

AMs consist of totipotent cells that have the ability to differentiate, specialize and to form organs (Clark, 1997). In angiosperms the cells of AMs have a tunica-corpus organization, which is translated into a similar organization in the organs that arise from it (Nozawa and Hirata, 2002). The tunica layer consists of two histogenic layers; histogenic layer I and histogenic layer II, while the corpus section comprises only one layer, histogenic layer III (Nozawa and Hirata, 2002). Each of these layers is responsible for the formation of specific regions within the developing bud sport. For example, histogenic layer I is responsible for the formation of the epidermis of flowers, stems, fruit and leaves, whereas the histogenic layer II is responsible for the formation of the sub-epidermal tissue, the cortex and the gametes (Burge *et al.*, 2002). Histogenic layer III produces the inner regions of a bud sport, which includes some of the cortex, the vascular tissues (phloem and xylem), the cambium and pith (Burge *et al.*, 2002).

The position of a mutation within the different histogenic layers is used to classify AM mutants (Poethig, 1987). Plant chimeras are divided into three main types; namely sectional chimeras, mericlinal chimeras and periclinal chimeras (Poethig, 1987) (Figure 1.6). A sectional chimera is an individual that possess the mutation in a section in all three histogenic layers, therefore, the mutant cell line is partially present in all layers (Burge *et al.*, 2002). A mericlinal chimera is an individual where the mutant cell line only occurs in a section of one of the histogenic layers and lastly, a periclinal chimera comes about when one or two histogenic layers consist entirely of the mutant genotype (Burge *et al.*, 2002).

Of the three types of chimeras, periclinal chimeras are of the most valued to the breeder as they are the most stable chimeras (Burge *et al.,* 2002). Mericlinal and sectional chimeras may become periclinal chimeras if the mutant cells have a selective advantage over the original genotype (Szymkowiak and Sussex, 1996). If not, the mutant cells are often out-grown over time by the original genotype cells, causing mericlinal or sectional chimeras to revert to the original non-chimeric plant (Szymkowiak and Sussex, 1996).

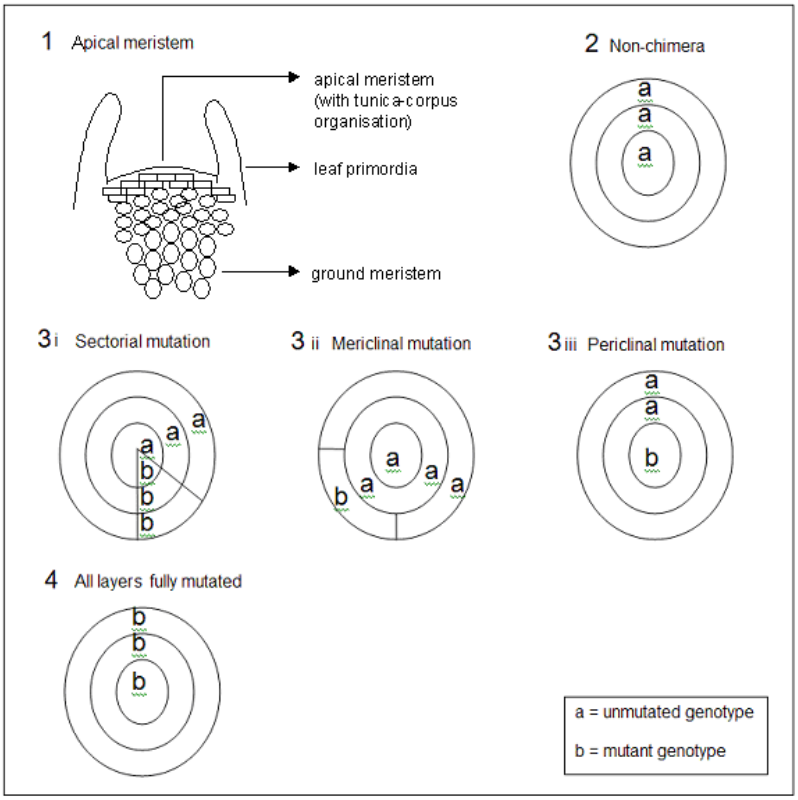

Figure 1.6 Diagrammatic representations of apical meristems and different mutations. 1. Side view of an apical meristem. Cross sections of apical meristems: 2. Non-chimera genotype. 3i. Sectional chimera genotype. 3ii. Mericlinal chimera genotype. 3iii. Periclinal chimera genotype. 4. Fully mutated genotype. (Cross sections adapted from Burge *et al.*, 2002).

1.6 POLYPLOIDY DETECTION

Once seed or tissue has been subjected to polyploidy induction, it is necessary to identify individuals or plant parts that have been successfully converted to polyploidy. Although the phenotypic effects of polyploidy are often visually apparent, these are usually not reliable methods of polyploidy detection (Dart *et al.*, 2004). Consequently, a variety of methods have been developed to detect the presence of polyploidy. These methods can be classified into two main groups; direct and indirect detection methods (Beck *et al.*, 2005).

1.6.1 Direct detection methods

Since polyploidy involves the duplication of the genomes and consequently an increase in the number of chromosomes within a cell, the most direct method and one of the oldest methods of polyploidy detection (Sharma and Sharma, 1965) involves quantifying the number of chromosomes (Vilhar *et al.*, 2002). Chromosomes are visualized and studied at metaphase or anaphase when they are most condensed and individually discernable (Darlington and La Cour, 1960). Chromosome counting therefore requires the study of tissue that is actively dividing, such as apical meristems of roots and shoots (Darlington and La Cour, 1960).

There are a number of limitations that are associated with chromosome counting (Beck *et al.*, 2003a). Some plant species, particularly forestry tree species, have numerous chromosomes that are relatively small making it difficult to make accurate counts (Hettasch, 1999; Beck *et al.*, 2003a; Negron-Ortiz, 2007). Other limitations that affect accurate counts of chromosomes include the limited number of dividing cells in the appropriate stage (Beck *et al.*, 2005). The counting of chromosomes is also perceived as a time consuming procedure (Bonos *et al.*, 2002; Negron-Ortiz, 2007), especially so for some tree species (Beck *et al.*, 2003a).

Another direct method is the quantification of DNA content using flow cytometry and has in recent years become a popular method to detect polyploidy (Dolezel *et al.*, 1992). This can be attributed to the refining of the buffers, the improved flow cytometry equipment and the accuracy of the method (Brummer *et al.*, 1999). DNA is quantified by staining the DNA with fluorochromes and then quantifying the DNA by the degree of fluorescence (Dolezel *et al.*, 1992). Flow cytometric quantification of DNA allows for the identification of chimeras, because very small quantities of DNA can be accurately measured (Awoleye *et al.*, 1994). Flow cytometry has been found to be generally a rapid method for the determination of ploidy, however expensive compared to more time consuming indirect methods of ploidy determination (Beck *et al.*, 2005).

1.6.2 Indirect detection methods

A number of alternative, more versatile, indirect methods to chromosome counting and flow cytometry have been developed for polyploidy detection (Beck *et al.*, 2005). The speed at which putative polyploids can be screened with many of the indirect methods have contributed to their popularity in recent years (Przywara *et al.,* 1988). These indirect methods used to detect polyploidy include the assessment of morphological characteristics (Morgan *et al.*, 2003), the determination of stomatal length and frequency (Beck *et al.,* 2003a), stomatal guard cell chloroplast frequency (Bingham, 1968) and arrangement (Beck *et al.,* 2003c). One other method requires more sophisticated equipment is the quantification of leaf chlorophyll content (Mathura *et al.,* 2006).

Assessment of morphological characteristics

Many morphological differences are apparent in the polyploid compared with its corresponding diploid; often these differences are sufficiently prominent to indicate successful induction of polyploidy (Hamill *et al.,* 1992). Some of these differences are apparent in flower size, pollen size, and leaf and root morphology (Hamill *et al.,* 1992). Growth retardation in seedlings is often a good indicator of polyploidy since polyploids are known for their reduced growth rates when compared with their diploid counterparts (Wright, 1962).

Polyploidy also tends to increase the size of plant organs. In some species, the flower size increases as the chromosome set number increases (Elliot, 1958; Kehr, 1996). Furthermore, because it has been shown that pollen size is positively correlated with ploidy level in many species (Blakeslee and Avery, 1932; Vilhar *et al.*, 2002), the measurement of pollen size was one of the earliest indirect polyploidy detection methods applied (Blakeslee and Avery, 1937). However, it may not be feasible for a breeder to use flower traits to detect polyploidy. The breeder will have to wait for the plant to flower, which may take years in the case of many tree species.

Leaves of polyploids also display a number of altered morphological characteristics when compared with the diploid. Leaves are often larger and thicker than the corresponding diploid (Frost, 1925; Vainola and Repo, 2001; Morgan *et al.*, 2003)). Interestingly, the leaves of polyploids are usually more rounded than those of the diploid (Thao *et al.*, 2003; Eeckhaut *et al.*, 2004). The ratio of length-to-width of the polyploid is thus increased, resulting in an increased leaf index (Hamill *et al.*, 1992; Ajalin *et al.*, 2003).

Roots of polyploids are often shorter and thicker than the diploid (Slade Lee, 1988; Hamill *et al.*, 1992). This is attributed to the reduced growth rate, where a greater amount of time is dedicated to cell division because of the increased chromosome number (Levin, 1983). The thicker roots on the other hand, are probably because of an increase in cell size (Slade Lee, 1988).

Because most of the morphological characteristics are quantitative in nature and subjected to environmentally induced phenotypic variation (Vandenhout *et al.*, 1995; Thao *et al.*, 2003), they cannot be universally applied to detect polyploidy and are generally deemed unreliable (Vandenhout *et al.*, 1995; Dart *et al.*, 2004).

Stomatal guard cell length and stomatal frequency

Stomatal guard cell length and stomatal frequencies have been successfully used to detect the presence of polyploidy in many plant species. The length of guard cells of stomata tends to increase as the ploidy level increases (Przywara *et al.,* 1988). With regards to the number of stomata, it has been shown that a negative correlation may exist between stomatal frequency and ploidy level (Tan and Dunn, 1973). The stomatal frequency is thus higher in diploids than in their tetraploids counterparts (Beck *et al.*, 2003b).

Stomatal chloroplast frequency and arrangement

Bingham (1968) showed that, with alfalfa, the number of chloroplasts in stomatal guard cells increased with an increase in ploidy level. Although not used as a detection method, it has been noted that with an increase in the number of

chloroplasts, the arrangement of chloroplasts is altered in the stomatal guard cells of the polyploidy (Beck *et al.*, 2003c). Typically in diploid stomatal guard cells the chloroplasts display a polarised pattern, while in the polyploid stomatal guard cells the chloroplasts are more evenly distributed along the periphery of the cell (Beck *et al.,* 2003c).

Nucleolar counts

Another indirect method of ploidy detection is that of nucleolar counting in interphase cells (Fankhauser and Humphrey, 1943; Vilhar *et al.*, 2002). Typically, one chromosome within a basic set of chromosomes within a genome possesses a nucleolar organizing region (NOR), often identifiable as a secondary constriction (Moscone *et al.*, 1995). This region houses, in tandem, rDNA genes, which code for ribosomes (Chen and Pikaard, 1997). During the transcription of these genes during interphase an intranuclear organelle forms, the nucleolus (Chen and Pikaard, 1997). Thus, a typical diploid nucleus will display a maximum of two nucleoli, while the tetraploid counterpart may display up to four nucleoli (Marcon *et al.*, 2005). Interphase nucleoli of somatic tissue are stained and counted with a microscope at an appropriate magnification (Lima-Brito *et al.*, 1998; Vilhar *et al.*, 2002). An advantage of this method is that the cells studied need not be of meristematic origin; therefore cells must be non-dividing in interphase (Vilhar *et al.*, 2002). As no particular plant material is required for nucleoli studies coupled with the efficient staining process, nucleolar counts have been described as a quick screening process in detection of polyploidy (Vilhar *et al.*, 2002).

Conversely, it should be noted that nucleoli counting is not effective in all instances (Vilhar *et al.*, 2002). Nucleolar dominance has been shown to occur in allopolyploids and allodiploids, which possess two or more chromosome sets with different karyotypes (Chen and Pikaard, 1997; Vilhar *et al.*, 2002). In these species the rDNA genes of one genome may be expressed, while these genes are repressed in the other genome(s) (Chen and Pikaard, 1997; Lima-Brito *et al.*, 1998). For example, in the case of an allotetrapolyploid displaying nucleolar dominance, a maximum of two nucleoli will be evident in the nucleus instead of a possible four.

In addition to nucleolar dominance, nucleolar fusion may take place when two or more nucleoli tend to fuse forming one large nucleolus (Jordan *et al.*, 1982). This event has been commonly observed in polyploids with many sets of chromosomes (Fankhauser and Humphrey, 1943), making nucleolar counting an unreliable polyploidy detection tool.

Chlorophyll content

It had been noted in a number of instances that as ploidy level increases the chlorophyll concentration within the polyploid's leaves increases (Joseph *et al.*, 1981; Molin *et al.*, 1982; Baer and Schrader, 1985; Warner *et al.*, 1987; Warner and Edwards, 1989; Romero-aranda *et al.*, 1997) and has been identified as a method of detecting polyploidy (Mathura *et al.,* 2006). Recently, the quantification of chlorophyll concentration using chlorophyll absorbance was successfully applied to distinguish between diploid and tetraploid *A. mearnsii* (Mathura *et al.,* 2006). This method cannot be universally applied without prior testing, as it has been shown that there was no difference in chlorophyll concentration between polyploids and diploids of *Ricinus communis* L. (Timko and Vasconcelos, 1981).

Table 1.6 shows the extent of the usage of the different polyploidy detection methods that have been successfully employed to detect induced polyploidy.

Table 1.6 Methods that have been successfully used to detect polyploidy in various species.

Method of detection	Species and reference
Seedling heights	*Datura* (Blakeslee and Avery, 1937); *Citrus* (Slade Lee, 1988).
Flower size	*Datura* (Blakeslee and Avery 1937); *Carica papaya* (Hofmeyr and van Elden, 1942); *Exacum* (Semeniuk, 1978).
Pollen size	*Carica papaya* (Hofmeyr and van Elden, 1942) *Bromus inermis* Leyss (Tan and Dun, 1973); *Exacum* (Semeniuk, 1978); *Dactylis glomerata* (Vilhar *et al.*, 2002); *Rosa chinensis minima* (Sims) Voss (Zlesak *et al.,* 2005).
Root length and diameter	*Citrus* (Slade Lee, 1988); *Musa acuminata* (Hamill *et al.*, 1992).
Stomatal length	*Bromus inermis Leyss* (Tan and Dun, 1973); *Actinidia deliciosa* (Przywara *et al.,* 1988); *Musa acuminata* (Hamill *et al.,* 1992; van Duren *et al.*, 1996); *Acacia mearnsii* (de Wild) (Beck *et al.,* 2003b); *Rosa chinensis minima* (Sims) Voss (Zlesak *et al.,* 2005).
Stomatal frequency	*Acacia mearnsii* (Beck *et al.,* 2003b); *Betula papyrifera* (Li *et al.,* 1996).
Chloroplast number	Cotton (Chaudhari and Barrow, 1975); Chicory (Rambaud *et al.*, 1992); Watermelon (Compton *et al.,* 1999); *Acacia mearnsii* (Beck *et al.*, 2005).
Nucleolar frequency	*Dactylis glomerata* (Vilhar *et al.,* 2002).
Chlorophyll concentration	*Festuca arundinacea* Schreb. (Joseph *et al.*, 1981); Alfalfa (Molin *et al.*, 1982); *Zea mays* (Baer and Schrader, 1985); *Panicum virgatum* (Warner *et al.*, 1987); *Atriplex confertifolia* (Warner and Edwards, 1989); *Citrus sinensis* (L.) and *Citrus limon* (L.) (Romero-aranda *et al.*, 1997); *Acacia mearnsii* (de Wild) (Mathura *et al.,* 2006).
DNA quantification (using flow cytometry)	Alfalfa (Brummer *et al.*, 1999); *Agrostis* (Bonos *et al.*, 2002); *Humulus lupulus* L. (Beatson *et al.,* 2003); *Arabidopsis* (Dart *et al.*, 2004); *Rosa* (Khosravi *et al.*, 2008).

1.7 POLYPLOIDY IN FORESTRY TREE BREEDING

As expected, polyploidy also occurs naturally in forestry tree species (Libby *et al.*, 1969). The highest prevalence of natural polyploids in tree species is found in the angiosperms (Wright, 1962), where it has been observed in a number of genera, for example *Betula*, *Acacia* and *Populus* (Wright, 1962). However in comparison with the frequency of non-woody polyploidy angiosperm species, natural polyploidy in tree species is uncommon (Gustafsson, 1960). Stebbins (1950) proposed that the small sized cambium initials are unable to contain the increase in genetic material, thus explaining the low frequency of angiosperm woody polyploids compared with non-woody polyploids (Gustafsson, 1960). Similarly, gymnosperms have been described as been “cytologically constant”, implying that the occurrence of natural polyploidy is very rare (Khoshoo, 1959; Gustafsson, 1960).

The finding of the triploid aspen (*Populus tremula*) in 1936 conjured much interest in polyploidy in forestry trees, as this tree exhibited an increased growth rate and favourable wood properties (Einspahr *et al.*, 1963; Libby *et al.,* 1969). It also possessed a higher pest and pathogen resistance than diploid aspen (Libby *et al.*, 1969). Since the identification of these desirable polyploid trees, programmes to research the cultivation of polyploidy trees have been established in a number of countries (Eriksson *et al.*, 2006). Experimental conversion of diploids to polyploids has been undertaken around the world in tree species from the genus *Pinus* (Mergen, 1959), *Eucalyptus* (Janaki *et al.*, 1969; Kampoor and Sharma, 1985) and *Acacia* (Moffet and Nixon, 1960; Blakesley *et al.*, 2002).

Mergen (1959) induced polyploidy in slash pine, loblolly pine, Austrian pine and Mugo pine. The polyploid individuals were, however, abnormal and did not show the expected desirable properties for cultivation (Wright, 1962; Mergen, 1959). Similar findings have been documented for other conifers; together with aberrant growth, high death rates were also recorded (Libby *et al.*, 1969). It is generally accepted that the potential of polyploid induction and gains in gymnosperms are poor (Wright 1962). Induced polyploid conifers also tend to revert back to their diploid state (Libby *et al.,* 1969).

In *Acacia* species polyploidy has been successfully induced in an attempt to confer infertility to curb excessive seed production (Moffett and Nixon, 1960; Beck *et al.*, 2005). Polyploidy has also been successfully induced in *Eucalyptus citriodora*, where the tetraploids displayed favourable phenotypes (Janaki *et al.*, 1969). More recently, polyploidy has also been induced in a hybrid of *Eucalyptus grandis* and *Eucalyptus tereticornis* to produce allopolyploids (Kampoor and Sharma, 1984). Table 1.7 provides a list of successfully induced polyploids in forestry tree species.

Table 1.7 Successfully induced polyploidy in commercial forestry tree species.

Species	Objective	Reference
A. mearnsii	Production of triploids to curb invasiveness; Increase in yield.	Moffett and Nixon (1960); Beck *et al.* (2003b)
A. dealbata	Production of triploids to curb invasiveness	Blakesley *et al.* (2002)
A. mangium	Production of triploids to curb invasiveness	Blakesley *et al.* (2002)
E. citriodora	Increase yield of essential oils	Janaki *et al.* (1969)
E. grandis × *E. tereticornis*	Production of allopolyploids; Increase in yield	Kampoor and Sharma (1984)

1.8 AIMS

Worldwide the demand for hardwood from commercial plantations is rising as consumption of forest products increases (Pijut *et al.*, 2007), in particular for *Eucalyptus* species, one of the most widely cultivated plantation species (FAO, 2001). Over the past forty years considerable effort has been wielded in conventional *Eucalyptus* breeding through crossing and selection strategies (Eldridge *et al.,* 1993). However, the long generation and reproductive cycle, and difficulty of controlled pollinations have led to the exploration of newer technologies (Pijut *et al.,* 2007).

The recognition of potential desirable phenotypic traits in polyploid trees has resulted in the inclusion of polyploidy induction in many tree breeding programmes (Elliot, 1958). In *Eucalyptus* it is expected that triploids produced from induced tetraploids crossed with diploids will display increased yield and reduced fertility. Although some research has been conducted on the induction of polyploidy in *Eucalyptus*, in depth knowledge about the techniques and long-term outcomes remain elusive.

This investigation was therefore undertaken to investigate the induction of polyploidy into different tissue types of *Eucalyptus* species and hybrids. The chemical inducer colchicine was used as it was more freely available than the other chemicals. Also, some institutional knowledge about its use was available. A number of detection methods were assessed for their ease of use and accuracy.

The main aims of this investigation were as follows:

- To induce tetraploidy in *Eucalyptus* species and hybrids using two recognized methods; seed induction and axillary bud induction.
- To establish polyploidy detection tools and to assess their applicability and accuracy in polyploidy detection in *Eucalyptus* and its hybrids.

CHAPTER 2
MATERIALS AND METHODS

2.1. INTRODUCTION

Polyploids often exhibit traits superior to that of their diploid progenitors (Levin, 1983). This led to the development of methodologies for the induction of polyploidy in economically important crops to harness the beneficial traits associated with polyploidy (Elliot, 1958). In crop improvement polyploidy has been extensively employed to increase yield (Elliot, 1958). However in forestry, in addition to an increase in yield, the use of polyploidy extends to decrease the fertility of invasive commercial forestry species, such as black wattle (Beck *et al.*, 2003).

Experiments involving artificial induction of polyploidy comprise an induction phase and a detection phase. These two phases compose the following three investigations in this research project (Figure 2.1):

Investigation 1

Polyploidy induction in *E. urophylla* and *E. grandis* through the treatment of seed with different colchicine concentrations (0.01%, 0.03% and 0.05%) and exposure times (18 and 24 hours).

Investigation 2

Polyploidy induction in *E. grandis* and a number of *Eucalyptus* hybrid clones through the treatment of axillary buds with different colchicine concentrations (0.5%, 1.0% and 1.5%).

Investigation 3

The detection of mixoploid and whole polyploid individuals from treated material (Investigations 1 and 2) through the counting of the number of leaf stomata and measurement of stomatal guard cell length; the determination of DNA content through flow cytometry; and the counting of the number of stomatal guard cell chloroplasts.

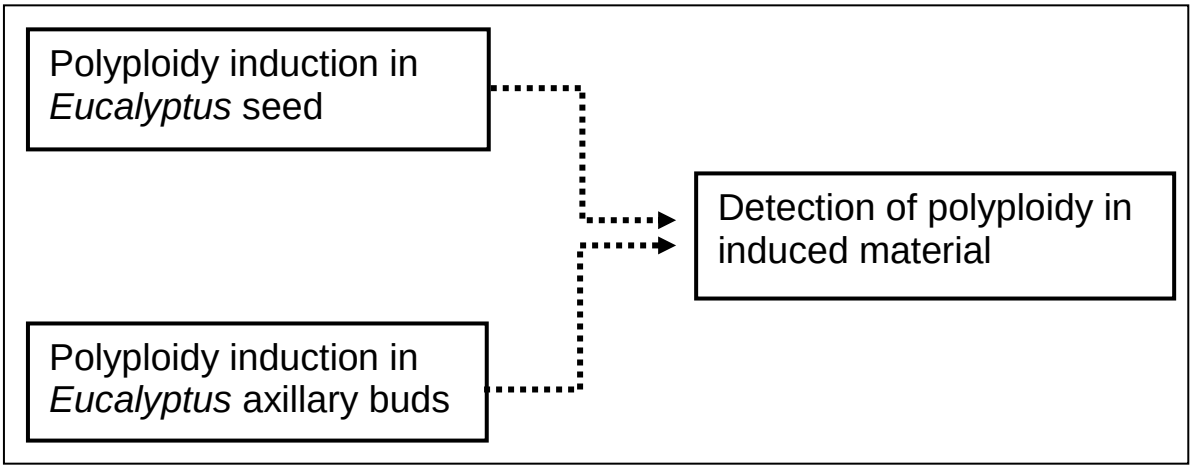

Figure 2.1 Summary of the investigations within this research project

2.2. MATERIALS

The plant material utilised in the different investigations of this research project comprised commercial material licenced to the Council for Scientific and Industrial Research (CSIR). *Eucalyptus* seed, as well young plants and hedge material of *Eucalyptus* clones were used.

2.2.1 Origin of seed

Seedlots, seed that originates from the same species, provenance and collection date, of two species namely, *E. urophylla* and *E. grandis* were supplied by the CSIR. The eight seedlots of *E. urophylla* and seven seedlots from *E. grandis* were sourced from trials located at Politzi in the Limpopo Province of South Africa and kept in storage at the CSIR in Nelspruit (Table 2.1).

Table 2.1 Seed source of *E. urophylla* and *E. grandis* species utilized in the induction of polyploidy in seed.

Species	Seed source	Latitude (°S)	Longitude (°E)	Altitude (m)	Rainfall (mm)
E. urophylla (8 seedlots)	Politzi, Limpopo Province, South Africa. The seed was collected from selections made from a provenance *cum* progeny trial.	23°46'	30°05'	760	1126
E. grandis (7 seedlots)	Politzi, Limpopo Province, South Africa. The seed was collected from selections made from a third generation breeding population originating from a progeny trial.	23°44'	30°06'	760	1342

2.2.2 Origin of axillary bud material

The induction of polyploidy in axillary buds involved the use axillary buds of clones (hedges and young plantlets with the same genotype propagated via cuttings). These clones consisted of two low-splitting *E. grandis* clones (SGR 1266 and SGR 1238) and four interspecific hybrids, one of which was an interspecific hybrid of *E. grandis* and *E. nitens* (G×N 075) and the remainder of the three clones were interspecific hybrids of *E. grandis* and *E. urophylla* (G×U 082, G×U 083 and G×U 111). The origin of the ortets, the mother trees from which the clonal material was derived in this investigation, is depicted in figure 2.2.

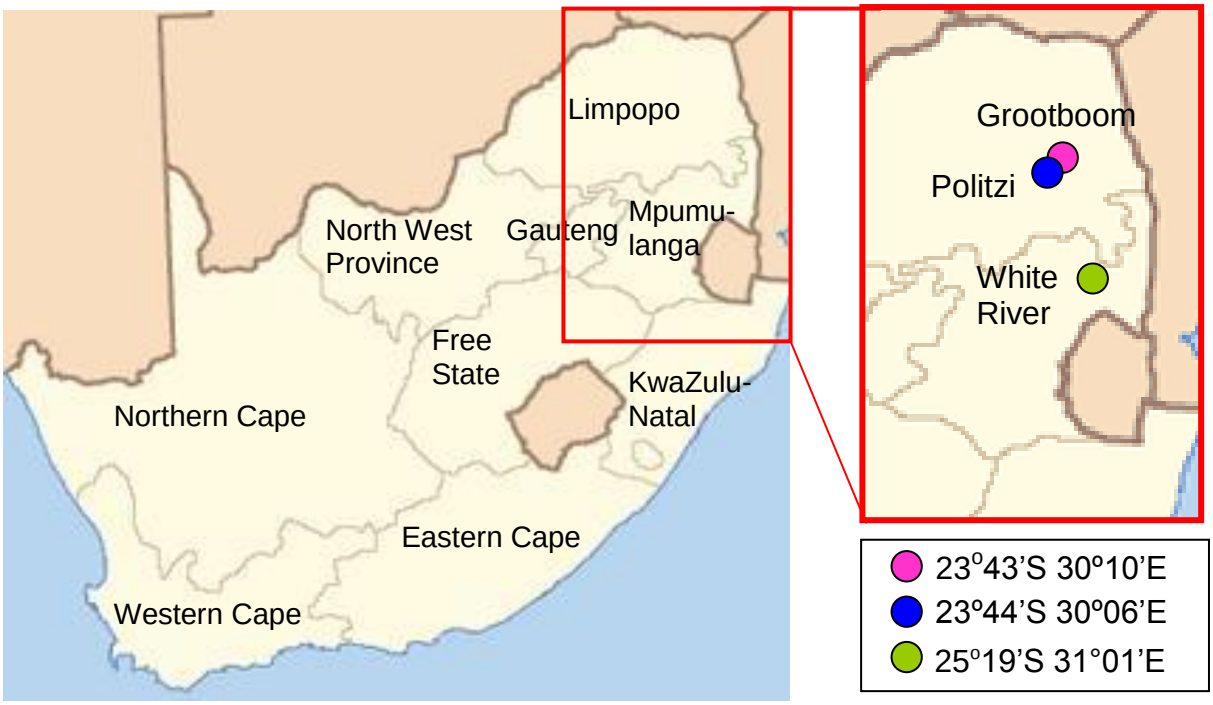

Figure 2.2 Origins of the ortets (adapted from Mercer, 2008).

The ortets of the clones of the two low-splitting *E. grandis* and four *E. grandis* hybrids that were used in this investigation originated from various locations in South Africa. The ortets of the two low-splitting *E. grandis* clones originated from Politzi in Limpopo Province. The ortet of the interspecific hybrid of *E. grandis* × *E. nitens* (G×N 075) clone originated from White River, Mpumulanga Province, while the ortets of the three different *E. grandis* × *E. urophylla* interspecific hybrid clones originated from Grootboom in the Limpopo Province (Table 2.2).

Ramets of the six clones were supplied for this investigation by Top Crop Nursery and Sunshine Seedling Services in Pietermaritzburg in KwaZulu-Natal and housed at the Institute for Commercial Forestry Research (ICFR) nursery, Pietermartizburg, KwaZulu-Natal. The ramets of the *E. grandis* clones were in hedge form in black plastic growth bags, while the ramets of the *E. grandis* interspecific hybrid clones were only a few months old and between 10 and 20 cm in height in seedling trays (Table 2.2). These young plantlets were immediately planted into black plastic growth bags containing commercial potting media.

Table 2.2 Origin of clones, supplier of ramets, and size and number of ramets.

Clone	Origin	Supplier	Number of ramets	Size
SGR1266	Politzi, Limpopo Province	Top Crop Nursery	6	Hedge
SGR1238		Top Crop Nursery	6	Hedge
G×N 075	White River, Mpumulanga Province	Top Crop Nursery	12	Plantlets: 15-20 cm
G×U 082	Groot Boom, Limpopo Province	Top Crop Nursery	12	Plantlets: 15-20 cm
G×U 083		Top Crop Nursery	12	Plantlets: 15-20 cm
G×U 111		Sunshine Seedling Services	12	Plantlets: 10 cm

A reference tetraploid clone (SFX 302) was included in investigation three (Figure 2.3). This reference clone was produced by Shell from an interspecific hybrid between *E. grandis* and *E. camaldulesis,* which was subsequently bought by the CSIR. Although many ramets have been propagated from the original ortet, the ramet selected for analysis in this investigation was housed at Northern Timbers in Politzi in the Limpopo Province. Cuttings were taken from the reference clone hedge in the afternoon, wrapped in wet tissue paper, sealed in a Zip Lock® plastic bag and couriered overnight to the University of KwaZulu-Natal, Pietermaritzburg, KwaZulu-Natal for analysis. Two ramets of the corresponding diploid clone (SFX 104) were acquired from Top Crop Nursery in Pietermaritzburg for analysis and housed in the nursery of the ICFR.

Figure 2.3 SFX 302 in the nursery at Northern Timbers (Hans Merensky Timbers) in Politzi in the Limpopo Province.

The hedges and young plantlets, including SFX 104 ramets, were watered once every two days for 20 minutes. They were fertilized once every two weeks with Gromor® 3:1:3 NPK fertilizer.

2.3 METHODS

As discussed above, the research can be divided into three different investigations, where the first investigation comprised the induction of polyploidy in seed, the second investigation the induction of polyploidy in axillary buds and the third the detection and verification of polyploidy.

Recipes of solutions are given in the appendix.

2.3.1 Investigation 1: Induction of polyploidy in seed

The experimental design of this investigation involved treating the eight *E. urophylla* seedlots and seven *E. grandis* seedlots with different concentrations of colchicine. The concentrations and time exposure of colchicine as well as the technique of induction were adapted from the methodology used for the induction of tetraploidy in *Acacia mearsii* (Beck *et al.*, 2003b) and from the induction of tetraploidy in *A. mangium* (Harbard, personal communication, 2006*).

Each polyploid induction treatment (specific concentration of colchicine and exposure time) consisted of two seeds per seedlot, repeated eight times (Figure 2.4). The treatment of the seed comprised the following steps: cleaning of seed, sterilisation of seed, polyploidy induction with colchicine and growth and maintenance of seedlings.

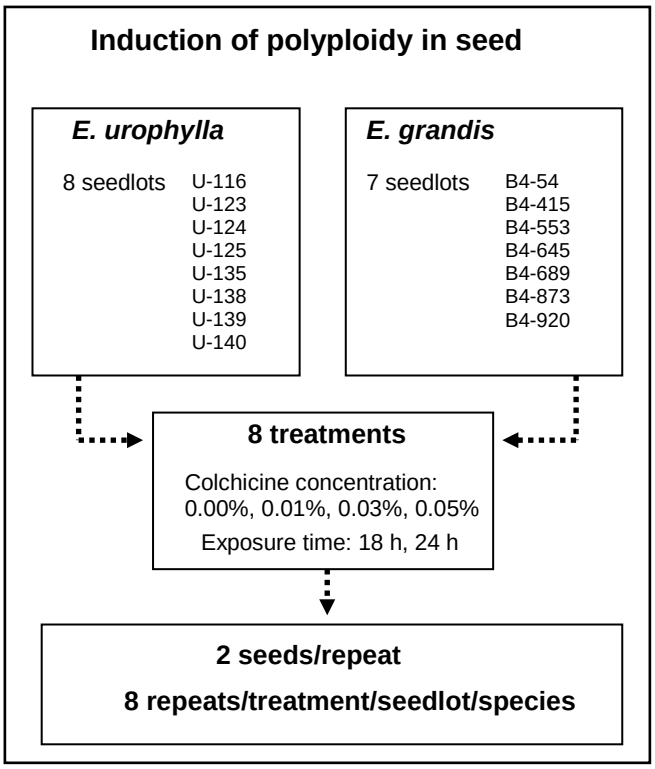

Figure 2.4 Experimental design for the induction of polyploidy in seed

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Cleaning of seed

The seedlots received were unclean, containing chaff, and required cleaning before treatment with colchicine (Figure 2.5). A sufficient amount of seed of each seedlot was cleaned manually for all experiments by separating the chaff from the seed. Seed of each seedlot was placed in airtight tubes, labelled and stored in a cool dark cupboard until use.

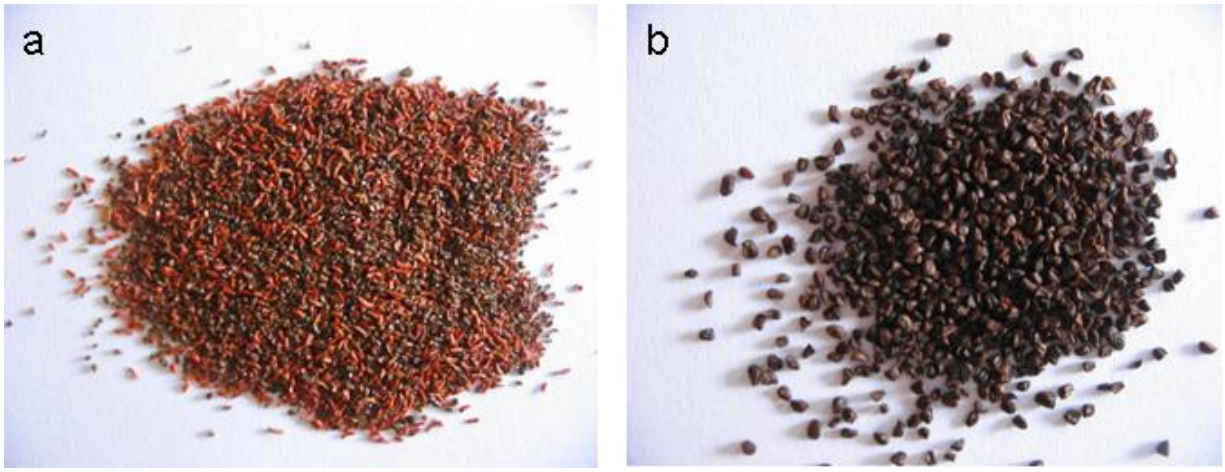

Figure 2.5 *Eucalyptus* seed. a. Uncleaned *E. grandis* seed containing chaff. b. cleaned *E. urophylla* seed.

Sterilisation of seed

Before the colchicine treatment of the seed commenced, the seed was first sterilised to destroy any fungal spores on the seed coat that may affect germination in the Petri dish. A sterilisation solution of 50% Jik® (1.75% active ingredient Sodium Hypochlorite ,$NaOCl^-$) was prepared. Seed was placed in a 50 ml beaker containing 10 ml of sterilisation solution and left for 10 minutes. Thereafter, the sterilisation solution was decanted and the seed rinsed three times with approximately 50 ml of distilled water per rinse.

Colchicine treatment of seed

Prior to the treatment of the seed, colchicine solutions of 0.01%, 0.03% and 0.05% were prepared from a 1% colchicine stock solution. For the control treatment, the colchicine solution was substituted with distilled water (also referred to as 0.00% colchicine). The seed was treated in prepared Petri dishes, each containing one layer of 90 mm Whatman® filter paper. The Petri dishes were labelled specifying seedlot, repeat, colchicine concentration and the exposure time to the colchicine solution.

Two seeds were placed into each Petri dish (one repeat of a treatment). Once the seeds had been placed in the appropriate dishes, 4 ml of the appropriate colchicine solution was added to the Petri dish. This was done in minimal light, as colchicine is light sensitive. The Petri dishes were then placed in a Heraeus incubator at 25^{o}C for the specified exposure time (18 or 24 hours). The Petri dishes were then removed from the incubator and the seeds rinsed three times with approximately 50 ml distilled water per rinse. The seeds were then placed into newly prepared Petri dishes, each prepared with one layer of filter paper moistened with distilled water, using a pair of forceps. These Petri dishes were left on the laboratory bench top (at approximately 25 ^{o} C), where they were exposed to light, to recommence germination for two weeks.

Growth and maintenance of seedlings

After two weeks, seedlings that possessed cotyledons were planted in 128s, polystyrene seedling trays, containing *Eucalyptus* seedling media (50% bark: 50% peat moss) obtained from Top Crop Nursery in Pietermartizburg. At the same time as planting, the root length and the shoot length of each seedling was measured using a small ruler. Each treatment was labelled with the seedlot number, colchicine concentration, exposure time (for example U-123 0.03 18). The seedling trays were kept at the Botany Gardens green house of the University of KwaZulu-Natal in Pietermaritzburg covered by black shade cloth (50% cover) and watered daily once every eight minutes for six seconds from 09:00 to 15:00. After six months, the water dosage was increased, and the seedlings were watered every eight minutes for ten

seconds. For the first two months, Nitrosol liquid fertilizer was applied once a week through a soil drench to the seedlings as well as Previcur® systemic fungicide to prevent damping-off. Thereafter, the seedlings were fertilized once every two weeks. At two months of age the number of surviving seedlings per treatment was measured and at seven months of age the number of atypical seedlings per treatment was also measured.

2.3.2 Investigation 2: Induction of polyploidy in axillary buds

The experimental design of this investigation involved treating axillary buds of *E. grandis* and *E. grandis* hybrid clones with different concentrations of colchicine. The range of colchicine concentrations (0.0%, 0.5%, 1.0% or 1.5%) selected for axillary bud induction was based upon the successful induction of polyploidy in *A. mangium* using these same concentrations and the exposure time period of three consecutive days (Harbard, personal communication, 2006).

For each polyploid induction treatment (specific concentration of colchicine) consisted of twenty axillary buds per clone (Figure 2.6). The treatment of the axillary buds comprised the following steps: multiplication of axillary buds, colchicine treatment of axillary buds and growth and maintenance of plants.

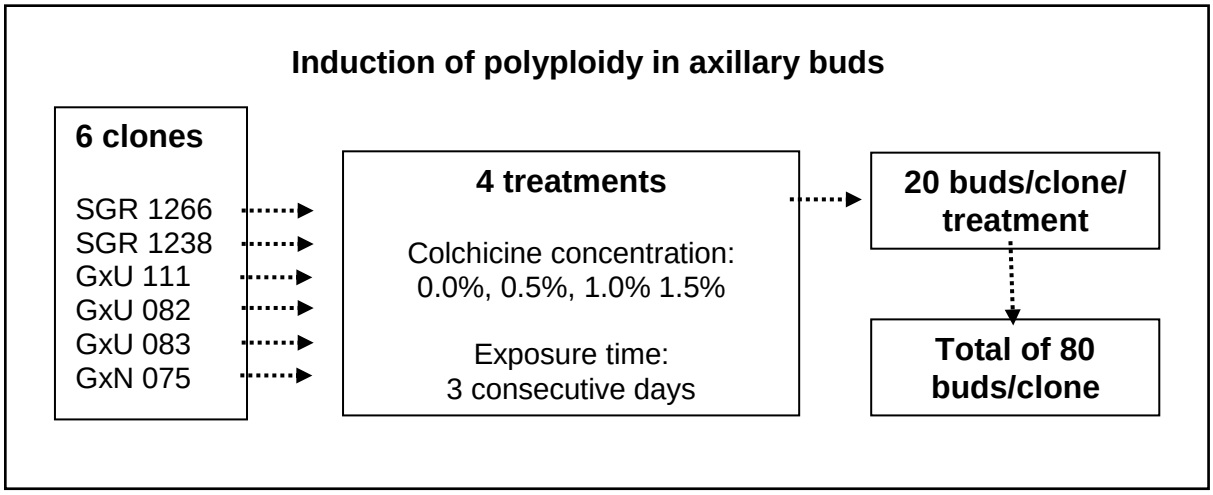

Figure 2.6 Experimental design for the induction of polyploidy in axillary buds

Multiplication of axillary buds

The ramets of the two *E. grandis* clones that were fully established in hedges at Top Crop Nursery and Sunshine Seedling Services were pruned regularly to stimulate and maintain lateral growth. On the other hand, the younger ramets of the *E. grandis* interspecific hybrid clones that were not established as hedges did not bear many axillary buds and were thus left to grow for a further four months. During this period these plants were continuously cut back to promote lateral growth and to increase the number of axillary buds available for the experiment.

Colchicine treatment of axillary buds

Once a sufficient number of axillary buds were obtained on the hedges and young plants, at least one axillary bud per branch and 80 or more in total per genotype, the branches were prepared for the colchicine treatment. Each branch was cut back to the first set of axillary buds to ensure axillary bud growth after treatment. Only the smallest axillary buds were treated with colchicine, as these contained the least number of cells within the meristematic tissue of the apical dome increasing the probability of inducing polyploidy in all histogenic layers. Figure 2.7 depicts the process of branch and axillary bud preparation for colchicine treatment.

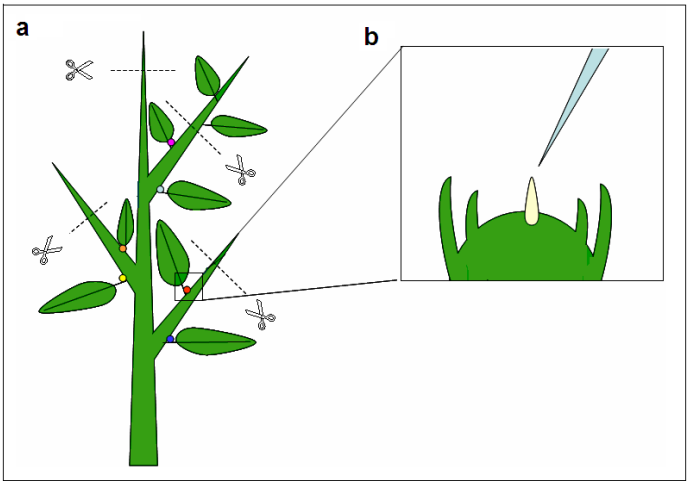

Figure 2.7 Preparation of plants for colchicine treatment of axillary buds. a. Preparation of the whole plant. b. Application of colchicine to an axillary bud.

The selected axillary buds were treated with one of four concentrations of colchicine; 0.0, 0.5, 1.0 or 1.5% colchicine. Five microlitres of the appropriate colchicine concentration was dropped on each of the 20 selected axillary buds per treatment per clone once daily during the early morning for three consecutive days. Treated axillary buds were labelled with a piece of cardboard tied to the stem below the bud, indicating the bud number and colchicine concentration. During the three days of treatment the clones were kept in the laboratory at a fairly constant humidity away from the nursery-watering regime to prevent the buds from getting wet and the colchicine solution washing away. After the three days of colchicine treatment the clones were returned to the ICFR nursery.

Growth and maintenance of plant

The treated axillary buds were then left to grow lateral branches on the plants for two months, after which the number of emergent bud sports were counted and the length of emergent bud sports were measured and assessed for any phenotypic aberrations. At six months the polyploidy detection commenced using stomatal guard cell length measurements. Throughout the six-month period lateral branches that grew from untreated axillary buds were removed to ensure growth of the colchicine treated axillary buds.

2.3.3 Investigation 3: Detection of polyploidy in the induced material

The detection of polyploidy in the seedlings and bud sports grown from the colchicine treated seed and axillary buds respectively, involved the use of various detection methods. All seedlings and bud sports were subjected to pre-screening using stomatal guard cell length measurements. Seedlings that displayed significantly ($p<0.05$) greater differences in stomatal guard cell measurements when compared with the controls were labeled as putative polyploids and subjected to flow cytometry to confirm the ploidy level. The confirmed tetraploids identified from flow cytometry were then subjected to stomatal frequency and stomatal chloroplast frequency measurements.

In the case of the bud sports, the stomatal guard cell length measurements of some leaves revealed areas of diploid type measurements and areas of tetraploid type measurements that were significantly ($p<0.05$) different from that of the controls; indicative of putative mixoploids. Thus, due to the small size of the polyploid areas, further polyploidy assessments of these putative mixoploids only involved DNA content quantification using flow cytomety.

The reference tetraploid (SFX 302) was also included in the assessment process to provide a measure of comparison for validation of polyploidy induction. Its tetraploid nature was discerned by the measurement of stomatal guard cell length, determination of stomatal frequency, determination of DNA content using flow cytometry and the determination of stomatal guard cell chloroplast frequency and arrangement.

Determination of stomatal guard cell length

The length of leaf stomata guard cells were measured on imprints of the abaxial side of intact leaves. These imprints were created by applying a layer of transparent nail varnish to the abaxial layer of the leaf. The nail varnish was left to dry for 10-15 minutes, after which the nail varnish was gently peeled off from the leaf surface. The nail varnish imprint was then mounted on a slide and covered with a coverslip. Nail varnish was then dropped onto the corners of the coverslip to attach the coverslip to the slide and to flatten the stomatal guard cell imprint for easy viewing and accurate measuring. The nail varnish imprints were then viewed with 40 × magnification using an Olympus Provis AX70 light microscope and the images captured by an Olympus camera attached to the microscope. The lengths of stomata guard cells on the images were measured using analySIS® 3.0 © 2002 Soft Imagining System GmbH.

Ten stomata per leaf of a total of eight leaves were measured (two leaves/branch, four branches) for the reference tetraploid, SFX 302, and its corresponding diploid SFX 104, to provide reference information.

Although a large number of seeds and axillary buds were induced; because of time constraints, a subset of seedlings and bud sports for each treatment were selected

for pre-screening using stomatal guard cell measurements. For the pre-screening of seedlings, seedlings that exhibited distinct morphological alterations were selected. This included three seedlings of each of the induction treatments (including the controls) of all seedlots of both species. Each seedling was labelled with the seedlot number, colchicine concentration, exposure time and seedling number (U-123 0.03 18 P1). Six stomatal measurements repeated three times, on two leaves per seedling, were performed (36 measurements per seedling). In contrast, bud sports did not exhibit distinct morphological alterations; therefore six bud sports per genotype per treatment were randomly selected for pre-screening. Two leaves were analysed per bud sport, where six stomata were measured per repeat and three repeats per leaf. The number of seedlings and bud sports selected for pre-screening using stomatal guard cell measurements are provided in Tables 2.3 and 2.4.

Table 2.3 Number of seedlings selected for pre-screening using stomatal guard cell measurements

Seedlings				
Species	**# of seedlings selected**	**# of treatments***	**# of seedlots**	**Total # of seedlings selected***
E. urophylla	3	6	8	144
E. grandis	3	6	7	126
Total				270

*excluding controls

Table 2.4 Number of bud sports selected for pre-screening using stomatal guard cell measurements.

Bud sports			
Genotypes	**# of bud sports selected**	**# of treatments***	**Total # of bud sports selected***
SGR 1238	6	3	18
SGR 1266	6	3	18
G×U 082	6	3	18
G×U 083	6	3	18
G×U 111	6	3	18
G×N 075	6	3	18
Total			108

*excluding controls

Determination of DNA content using flow cytometry

In the preparation of the samples for the quantification of DNA content using flow cytometry, leaf material was treated mechanically and chemically to disrupt the cells to release intact nuclei. The DNA contained in the nuclei was then stained with a fluorescent dye and then quantified by the flow cytometry.

Sample preparation for flow cytometry involved the use of the Otto two step protocol (Otto 1990), which was slightly modified to achieve results with higher accuracy. Intact nuclei were extracted from leaf cells by first placing 50 mg of leaf material in one millilitre (ml) of ice cold Otto I buffer containing 80 mg of insoluble Polyvinylpyrrolidone 40 (PVP 40). Thereafter the leaf material was chopped with a razor blade to form a fine pulp. The pulp was subsequently filtered through a fine mesh of 55 µm and centrifuged at 1200 rpm for five minutes, after which the supernatant was removed and discarded. A further 100 µl of Otto I buffer was then added to the pellet and the samples then incubated at room temperature for two to three hours. In the second step, 400 µl Otto II buffer and 400 µl of a 0.04 mg ml^{-1} Propidium Iodide (PI) solution was added to each of the incubated samples. The samples were then left in the dark for 10 minutes before analysis.

Flow cytometry analyses were performed on the seedlings and bud sports that were identified as putative polyploids or mixoploids using stomatal guard cell length measurements. At nine months of age two leaves, one leaf per branch from two branches, per seedling were subjected to flow cytometry to quantify the DNA content and confirm the presence of polyploidy. These mixoploids and tetraploids were planted into bags and pruned to encourage the growth of lateral branches. Once branches were forming, after planting and cutting back, the plants were retested to confirm the ploidy level; one leaf per branch from two branches were tested. To incorporate the use of a reference plant, the DNA content of one leaf from the diploid control of each mixoploid and tetraploid was used as an external standard and analysed using flow cytometry.

Before DNA content could be quantified, the Beckman Coulter Epics XL-MCL Flow Cytometer with a 488 nm laser was calibrated using Beckman Coulter Flow Check

fluorospheres. It is recommended that the half-peak coefficient of variance (HPCV) readings should be below 2.00% for the flow cytometer to be calibrated.

The relative DNA content of the population of nuclei from each leaf sample was displayed as a series of histograms on a computer screen coupled to the flow cytometer. The analysis of each sample was stopped once 5 000 nuclei (events) were measured or after 400 seconds had lapsed. In some instances where the peaks could not be clearly discerned due to debris in the sample, a further 5 000 nuclei were measured to achieve a result of greater accuracy. The ploidy level of the leaves measured was calculated according to the following equation (Dolezel *et al.*, 2007):

Sample ploidy =

$$\text{Reference ploidy (from external standard)} \times \frac{\text{mean position of the G1 sample peak}}{\text{mean position of the G1 reference peak}}$$

G1 = non-dividing state of a nucleus in the cell cycle containing single stranded chromosomes.

Determination of stomatal frequency

After the quantification of DNA content using flow cytometry, stomatal frequency was measured in the identified tetraploids. The nail varnish imprints from the stomatal guard cell measurements were also used to quantify the leaf stomatal frequency. Stomatal frequency was quantified by counting the stomata within a defined area (field of view) on the nail varnish imprints viewed with 20 × magnification using an Olympus Provis AX70 light microscope and the images then captured by an attached Olympus camera. The stomata on the images were 'touch' counted using analySIS® 3.0 © 2002 Soft Imagining System GmbH.

In the case of the reference tetraploid (SFX 302) and its corresponding diploid (SFX 104), six fields of view per leaf of eight leaves were counted (two leaves/branch, four branches). For the tetraploid seedlings identified and their corresponding diploids, stomata were counted in six fields of view of two leaves per seedling.

Determination of stomatal chloroplast frequency

Stomatal chloroplasts were counted in the stomata of the abaxial epidermal layer of the leaf. Small sections of the abaxial epidermal layer were peeled off from each leaf with a sharp razor blade and placed on a slide. A drop of iodine stain was then dropped onto the epidermal peels. A coverslip was then placed over the iodine stain on the epidermal peels. The stomata were then viewed with 100 × magnification using an Olympus Provis AX70 light microscope using oil emersion and the images captured by an attached Olympus camera. The chloroplasts in the stomatal guard cells on the images were then 'touch' counted using analySIS® 3.0 © 2002 Soft Imagining System GmbH. When viewing live images of the stomata, it was necessary to toggle the focus knob of the microscope to count all the chloroplasts as the chloroplasts lay on different planes within the stomata.

The stomatal chloroplast analysis of the reference tetraploid (SFX 302) and its corresponding diploid (SFX 104) involved the counting of chloroplasts of six stomata per leaf in a total of eight leaves. The stomatal chloroplast frequency of four confirmed tetraploids was also measured. As this is a destructive method of sample preparation, only two leaves per tetraploid seedling and its corresponding diploid control were used for the counting of chloroplasts; 11 or 12 stomata were assessed on 3 separate slides, a total of 34 abaxial stomata assessed per leaf. The arrangement of the chloroplasts in the stomatal guard cells were also observed and noted as being either polarised or dispersed, to identify additional phenotypic alterations that could be associated with an increased chromosome number.

Detection of morphological differences between diploids, confirmed mixoploid and confirmed tetraploid seedlings

The phenotype of the confirmed mixoploids, confirmed tetraploids and corresponding diploids were compared to determine which morphological traits were good indicators of the conversion of diploidy to polyploidy. The traits which were observed were leaf length and width (leaf index), leaf size, colour of leaves, apical dominance, leaf margins.

2.4 STATISTICAL ANALYSIS

The data were captured and stored in Microsoft Excel (2003) spreadsheets. Microsoft Excel was also used to create graphs and to export data into the statistical programmes SAS (SAS Institute Inc., 1990) and GenStat 11th Edition (Lane and Payne, 1996), which was employed for the various statistical analyses.

The statistical analyses that were performed included:

1. Basic statistics such as means, standard deviation and range were calculated for the different measurements undertaken, stomatal guard cell length and frequency, stomatal guard cell chloroplast frequency, to determine the variation in quantitative measurements between the treated material and controls.
2. F-tests were performed to determine which factors were responsible for the variation observed in the treated plant material when compared with the controls. These F-tests include a Generalized Linear Mixed Model (GLMM), which was performed on binomial data; and a Restricted Maximum Likelihood (REML) analysis, which was performed on unbalanced data.
3. The Bonferroni correction (a conservative *post hoc* test to an F-test), a multiple-comparison procedure, was used to determine significant differences between means of various parameters.
4. The Holm-Sidak test (a *post hoc* test to an F-test) to determine significant differences between means using the Holm-Sidak test statistic.
5. The Shapiro-Wilk test for normality was performed on certain data sets to test the normality of the distribution.
6. The Mann-Whitney U-test (a *post hoc* test to an F-test) was performed to test the significance between means in a dataset that defies a normal distribution.

CHAPTER 3
RESULTS: INDUCTION OF POLYPLOIDY

3.1 INTRODUCTION

Both seed and axillary buds have been popular sources of plant material in polyploidy induction experiments, because in many species these sources of material display relatively high conversion rates from diploidy to polyploidy (Dermen, 1940). Both seed and axillary buds exhibit advantages and disadvantages as sources of plant material for polyploid induction in forestry trees. Seed induction is convenient, because a large number of seed can be treated simultaneously. However, the genotypes of the seed are unknown and the resulting phenotypes are diverse and unpredictable. The diverse nature of seed induced polyploidy does provide an opportunity to select the best performing polyploid. On the other hand, axillary buds provide the opportunity to mass produce polyploids that are clonal in nature. *Eucalyptus* clones may comprise interspecific hybrids, allowing for the formation of allopolyploids or segmental allopolyploids, thus polyploidy induction through axillary buds would retain these hybrid genotypes, but in duplicated state.

Polyploidy induction in forestry species and hybrids could be applied according to needs, either by inducing polyploidy into seed, allowing for genetic variation, or into axillary buds allowing for clonal propagation of polyploids.

3.2 INVESTIGATION 1: INDUCTION OF POLYPLOIDY IN SEED

The effects of different concentrations of colchicine (0.00, 0.01, 0.03, 0.05%) applied for either 18 h or 24 h to *E. urophylla* and *E. grandis* seed was investigated. The effects were evaluated by assessing the growth response of the seedlings by measuring their root and shoot lengths, the survival of the seedlings and the number of atypical seedlings that survived. For *E. urophylla* eight seedlots and seven for *E. grandis* were used. Two seeds were included in each treatment and replicated eight

times per seedlot, making a total of 16 treated seeds per treatment for both *E. urophylla* and *E. grandis*. Figure 3.1 illustrates the timeline of the induction process.

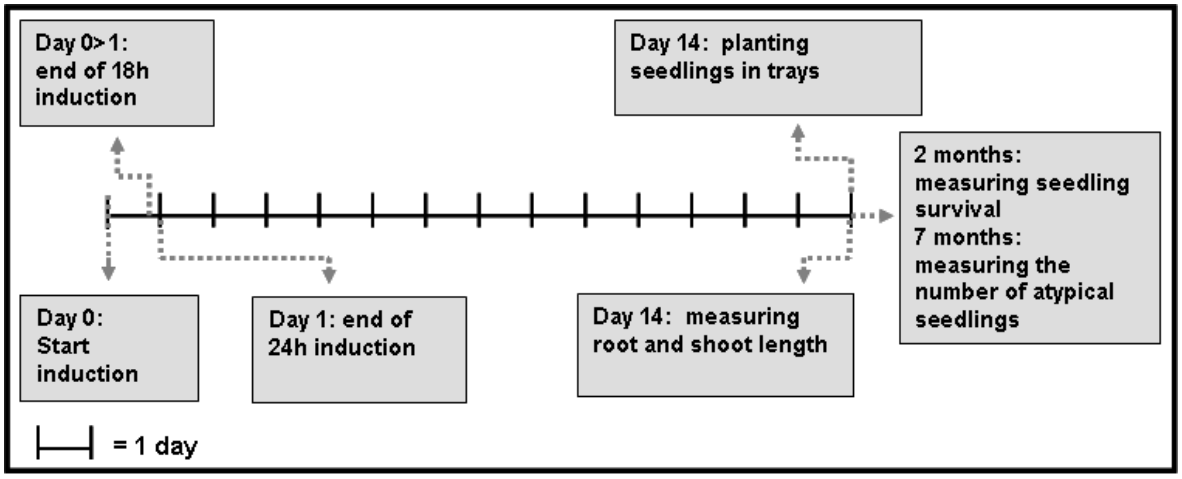

Figure 3.1 Timeline illustrating the induction phase and seedling evaluation phases.

3.2.1 Root and shoot length of germinated treated seed

The effect of colchicine on root and shoot growth of the seedlings was determined by measuring the length of the roots and shoots two weeks after colchicine treatment prior to the planting of the young seedlings into polystyrene trays. The seed of *E. urophylla* and *E. grandis* had been germinated in different concentrations of colchicine (0.00%, 0.01%, 0.03% and 0.05%) for either 18 or 24 hours.

Generally, it was observed that the roots and shoots of colchicine treated seed of both *E. urophylla* and *E. grandis* displayed reduced growth and swollen hypocotyls (Figure 3.2).

Figure 3.2 Colchicine treated and untreated *E. urophylla* seedlings after 24 hours exposure to colchicine. a. 0.05% colchicine. b. 0.00% colchicine (control).

E. urophylla

The effect of different colchicine concentrations for either 18 or 24 h of time exposure on root and shoot growth for the different *E. urophylla* seedlots was compared (Table 3.1). There were marked differences between the performances of the different seedlots over all treatments. Generally the measurements were smaller for root and shoot length in the treated seed showing the stunting effect of colchicine. When the root and shoot performances of the different seedlots were ranked within treatments, seedlot U-116 outranked many other seedlots in shoot length and root length in most treatments, displaying the least sensitivity to colchicine of all the seedlots. A contrasting trend could be established for seedlot U-123, which displayed the greatest shoot growth retardation in all treatments and root growth of most treatments.

Table 3.1 **Mean root lengths (cm) and mean shoot lengths (cm) recorded for different treatments for different *E. urophylla* seedlots.**

Seedlot	colchicine concentration (%) and exposure time (h)								Range
	Control 18	Control 24	0.01 18	0.01 24	0.03 18	0.03 24	0.05 18	0.05 24	
	E. urophylla root length								
U-116	1.88 ± 0.49	1.34 ± 0.42	1.49 ± 0.56	1.62 ± 0.51	1.54 ± 0.56	1.53 ± 0.46	1.30 ± 0.46	1.20 ± 0.32	0.68
U-123	1.52 ± 0.44	1.53 ± 0.51	1.24 ± 0.44	0.94 ± 0.49	0.98 ± 0.36	0.57 ± 0.22	0.63 ± 0.40	0.29 ± 0.20	1.24
U-124	1.68 ± 0.54	1.39 ± 0.59	1.11 ± 0.49	1.13 ± 0.38	1.11 ± 0.47	1.26 ± 0.24	0.82 ± 0.37	1.05 ± 0.30	0.86
U-125	1.99 ± 0.61	1.52 ± 0.38	1.59 ± 0.43	1.05 ± 0.32	0.90 ± 0.48	0.88 ± 0.31	1.04 ± 0.52	0.52 ± 0.19	1.47
U-135	1.22 ± 0.40	1.64 ± 0.60	1.04 ± 0.32	1.13 ± 0.54	0.97 ± 0.26	1.28 ± 0.48	1.28 ± 0.57	1.26 ± 0.32	0.29
U-138	1.24 ± 0.43	1.13 ± 0.39	1.13 ± 0.36	1.00 ± 0.31	1.00 ± 0.48	0.99 ± 0.33	0.86 ± 0.29	0.63 ± 0.25	0.62
U-139	1.23 ± 0.48	1.30 ± 0.45	1.36 ± 0.44	1.04 ± 0.38	1.01 ± 0.55	0.98 ± 0.39	0.84 ± 0.39	0.88 ± 0.52	0.53
U-140	0.98 ± 0.21	1.11 ± 0.37	0.96 ± 0.39	1.10 ± 0.29	0.94 ± 0.38	0.99 ± 0.31	0.78 ± 0.38	0.74 ± 0.41	0.36
	E. urophylla shoot length								
U-116	1.64 ± 0.23	1.58 ± 0.41	1.56 ± 0.43	1.68 ± 0.33	1.51 ± 0.51	1.63 ± 0.19	1.68 ± 0.50	1.62 ± 0.35	0.16
U-123	1.55 ± 0.38	1.60 ± 0.50	0.99 ± 0.21	0.79 ± 0.18	0.77 ± 0.23	0.68 ± 0.21	0.68 ± 0.21	0.60 ± 0.19	0.95
U-124	1.41 ± 0.40	1.24 ± 0.45	1.19 ± 0.53	1.13 ± 0.43	1.14 ± 0.40	1.13 ± 0.43	0.90 ± 0.23	1.01 ± 0.23	0.51
U-125	1.56 ± 0.24	1.61 ± 0.30	1.32 ± 0.25	1.06 ± 0.23	0.99 ± 0.43	0.91 ± 0.30	1.06 ± 0.32	0.69 ± 0.20	0.66
U-135	1.18 ± 0.21	1.29 ± 0.29	1.10 ± 0.31	1.20 ± 0.23	0.91 ± 0.26	1.10 ± 0.31	0.93 ± 0.23	1.04 ± 0.31	0.29
U-138	1.41 ± 0.25	1.20 ± 0.25	1.26 ± 0.29	1.08 ± 0.33	0.96 ± 0.25	1.07 ± 0.30	0.83 ± 0.20	0.91 ± 0.23	0.58
U-139	1.51± 0.36	1.61 ± 0.37	1.54 ± 0.20	1.33 ± 0.35	1.16 ± 0.50	1.23 ± 0.38	1.02 ± 0.38	1.16 ± 0.40	0.53
U-140	1.48 ± 0.34	1.39 ± 0.24	1.14 ± 0.26	1.37 ± 0.26	1.00 ± 0.35	0.98 ± 0.40	0.89 ± 0.36	0.84 ± 0.38	0.64

Red indicates the lowest mean root and shoot length within treatments and between seedlots
Blue indicates the highest mean root and shoot length within treatments and between seedlots

The overall effect of different colchicine concentrations for either 18 h or 24 h of exposure time displayed similar trends for roots and shoots. Generally, an increase in colchicine concentration resulted in the reduction of root and shoot growth for both 18 and 24 h of exposure (Table 3.2).

Table 3.2 Overall mean root lengths (cm) and mean shoot lengths (cm) for different treatments for all *E. urophylla* seedlots.

E. urophylla	**colchicine concentration (%) and exposure time (h)**							
	Control 18	**Control 24**	**0.01 18**	**0.01 24**	**0.03 18**	**0.03 24**	**0.05 18**	**0.05 24**
Root length	1.47 ± 0.56	1.37 ± 0.49	1.24 ± 0.47	1.13 ± 0.42	1.06 ± 0.48	1.06 ± 0.44	0.94 ± 0.48	0.82 ± 0.45
Shoot length	1.47 ± 0.33	1.44 ± 0.39	1.26 ± 0.37	1.20 ± 0.38	1.06 ± 0.43	1.09 ± 0.41	1.00 ± 0.43	0.98 ± 0.41

This general trend was also demonstrated by the negative slopes of the graphs in figure 3.3. When the effects of colchicine concentration at 18 h of exposure were compared with that of 24 h of exposure, the roots and the shoots exposed for 24 h were marginally more stunted in their growth when compared with those exposed for 18 h. For root growth, a colchicine concentration of 0.03% showed a similar growth response for 18 h and 24 h of exposure. However, with an increased colchicine concentration (0.05%), root growth was once again more stunted with 24 h of exposure to colchicine in comparison with 18 h of exposure. Shoot growth was equally stunted with an increased colchicine concentration with either 18 h or 24 h of exposure.

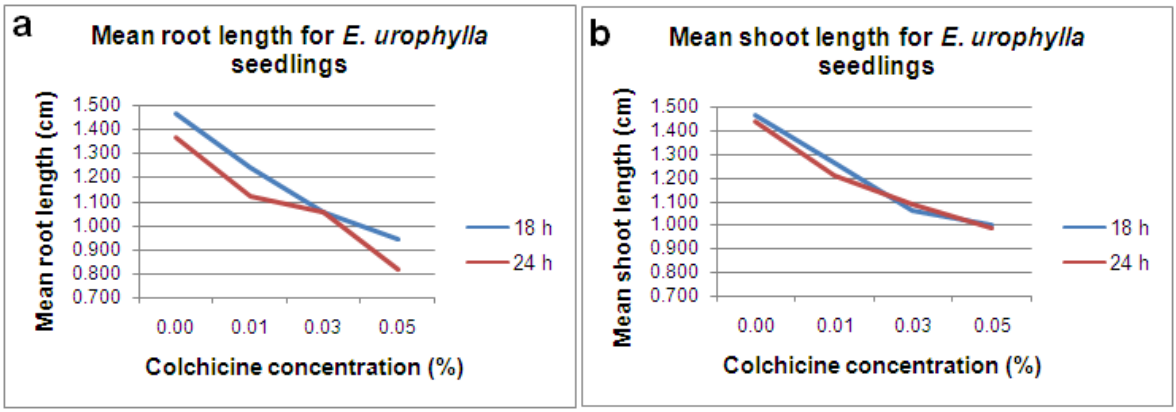

Figure 3.3 Root and shoot growth of seed exposed to different colchicine concentrations for 18 and 24 hours. a. root length (cm); b. shoot length (cm).

E. grandis

The effect of different colchicine concentrations for either 18 or 24 h of exposure time on root and shoot growth for different *E. grandis* seedlots was compared (Table 3.3). Generally the measurements were lower for root and shoot length in the treated seed showing the stunting effect of colchicine. Unlike *E. urophylla* there was no clear pattern of which seedlots root and shoot lengths were most severly affected. When the root and shoot performances of the different seedlots were ranked within treatments, seedlot B4-689 outranked all the seedlots in root length and shoot length in three of the strongest treatments (0.03 24, 0.05 18, 0.05 24), displaying the least sensitivity to colchicine. Seedlot B4-54 root and shoot growth measurements displayed the greatest range in root length between treatments and the greatest range in shoot length between treatments, displaying the greatest root and shoot growth retardation, thus indicating B4-54 was the most affected by colchicine concentration and exposure time.

Table 3.3 **Mean root lengths (cm) and mean shoot lengths (cm) recorded for different treatments for different *E. grandis* seedlots.**

Seedlot	colchicine concentration (%) and exposure time (h)								Range
	Control 18	Control 24	0.01 18	0.01 24	0.03 18	0.03 24	0.05 18	0.05 24	
	E. grandis root length								
B4-54	1.03 ± 0.37	0.93 ± 0.23	0.99 ± 0.51	0.81 ± 0.26	0.86 ± 0.40	0.63 ± 0.37	0.60 ± 0.32	0.29 ± 0.19	0.83
B4-415	1.12 ± 0.37	0.84 ± 0.20	0.86 ± 0.31	0.79 ± 0.25	0.73 ± 0.25	0.78 ± 0.34	0.79 ± 0.31	0.64 ± 0.29	0.38
B4-553	0.80 ± 0.33	0.64 ± 0.34	0.93 ± 0.22	0.65 ± 0.32	0.83 ± 0.20	0.56 ± 0.24	0.54 ± 0.23	0.43 ± 0.29	0.50
B4-645	0.92 ± 0.27	1.07 ± 0.44	0.98 ± 0.25	1.11 ± 0.67	1.11 ± 0.33	0.83 ± 0.26	0.86 ± 0.35	0.74 ± 0.36	0.38
B4-689	0.96 ± 0.38	0.78 ± 0.30	0.99 ± 0.26	0.96 ± 0.40	0.71 ± 0.28	0.89 ± 0.24	0.98 ± 0.33	0.95 ± 0.33	0.28
B4-873	1.07 ± 0.38	1.01 ± 0.36	0.73 ± 0.31	0.81 ± 0.31	0.74 ± 0.33	0.59 ± 0.33	0.39 ± 0.20	0.39 ± 0.15	0.68
B4-920	0.95 ± 0.30	0.84 ± 0.27	1.21 ± 0.29	0.90 ± 0.36	1.04 ± 0.42	0.76 ± 0.23	0.90 ± 0.34	0.89 ± 0.41	0.45
	E. grandis shoot length								
B4-54	1.37 ± 0.24	1.18 ± 0.18	1.03 ± 0.24	0.99 ± 0.26	0.96 ± 0.28	0.82 ± 0.34	0.63 ± 0.31	0.53 ± 0.21	0.68
B4-415	1.19 ± 0.22	1.15 ± 0.23	1.26 ± 0.31	1.16 ± 0.34	1.10 ± 0.26	0.88 ± 0.24	1.13 ± 0.31	0.84 ± 0.29	0.53
B4-553	1.03 ± 0.14	1.12 ± 0.27	1.13 ± 0.25	0.98 ± 0.29	0.99 ± 0.23	0.78 ± 0.29	0.96 ± 0.25	0.68 ± 0.30	0.44
B4-645	1.43 ± 0.26	1.58 ± 0.35	1.47 ± 0.27	1.26 ± 0.34	1.43 ± 0.32	1.17 ± 0.23	0.94 ± 0.41	1.10 ± 0.37	0.53
B4-689	1.35 ± 0.37	1.18 ± 0.31	1.43 ± 0.25	1.15 ± 0.27	1.14 ± 0.29	1.22 ± 0.26	1.23 ± 0.46	1.25 ± 0.35	0.26
B4-873	1.14 ± 0.23	1.26 ± 0.32	1.03 ± 0.27	1.08 ± 0.24	0.92 ± 0.24	0.77 ± 0.29	0.68 ± 0.29	0.59 ± 0.18	0.55
B4-920	1.19 ± 0.24	0.98 ± 0.14	1.36 ± 0.30	1.11 ± 0.29	1.11 ± 0.31	1.16 ± 0.30	1.19 ± 0.28	1.04 ± 0.36	0.32

Red indicates the lowest mean root and shoot length within treatments and between seedlots
Blue indicates the highest mean root and shoot length within treatments and between seedlings

When the overall effect of different colchicine concentrations, for either 18 or 24 h, on root and shoot growth was compared, it was found that, similarly to *E. urophylla*, as colchicine concentration increased so root and shoot growth decreased (Table 3.4).

Table 3.4 Overall mean root lengths (cm) and mean shoot lengths (cm) for different treatments for all *E. grandis* seedlots.

E. grandis	colchicine concentration (%) and exposure time (h)							
	Control 18	**Control 24**	**0.01 18**	**0.01 24**	**0.03 18**	**0.03 24**	**0.05 18**	**0.05 24**
Root length	0.98 ± 0.35	0.87 ± 0.33	0.96 ± 0.34	0.86 ± 0.40	0.86 ± 0.35	0.72 ± 0.31	0.72 ± 0.35	0.62 ± 0.38
Shoot length	1.24 ± 0.28	1.20 ± 0.31	1.24 ± 0.31	1.10 ± 0.30	1.09 ± 0.31	0.97 ± 0.33	0.96 ± 0.39	0.86 ± 0.39

The same general trend was observed in *E. grandis,* as in *E. urophylla*, where the seedlings exhibited more root and shoot growth retardation in the 24 h treatments compared with 18 h treatments for the same colchicine concentration. However, compared with *E. urophylla,* a larger difference existed in between exposure for 18 h and 24 h of exposure for both the root and the shoot growth (Figure 3.4).

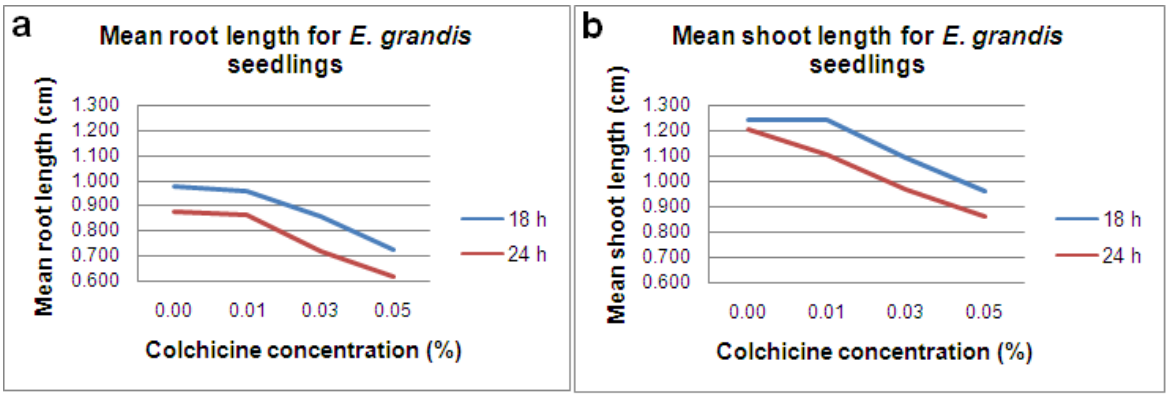

Figure 3.4 Root and shoot growth of seed exposed to different colchicine concentrations for 18 and 24 hours. a. root length (cm) and b. shoot length (cm).

E. urophylla and E. grandis

A Restricted Maximum Likelihood (REML) analyses and Holm-Sidak *post hoc* tests were performed on root length measurements and shoot length measurements for the both species combined. These analyses were performed to investigate the effects of species, colchicine concentration and exposure time on the root and shoot lengths of the seedlings.

The REML analysis performed on the root measurements for both species (Table 3.5) revealed that species ($p<0.01$) had a significant effect on root length indicating the mean root lengths of *E. urophylla* seedlings were significantly ($p<0.01$) different from the mean root lengths of *E. grandis* seedlings. Exposure time ($p<0.001$), colchicine concentration ($p<0.001$), as well as the interaction between species and colchicine concentration ($p<0.001$) also had a significant effect on the root length of the *E. urophylla* and *E. grandis* seedlings.

Table 3.5 REML analysis on the root length of *E. urophylla* and *E. grandis* seedlings.

Source of variation	Wald statistic	n.d.f.	F value	d.d.f.	p value
Species	15.78	1	15.78	13	0.002
Colchicine concentration	269.17	3	89.72	1891	<0.001
Time	27.77	1	27.77	1891	<0.001
Species × Colchicine concentration	34.7	3	11.57	1891	<0.001
Species ×Time	0.57	1	0.57	1891	0.449
Colchicine concentration ×Time	1.11	3	0.37	1891	0.775
Species × Colchicine concentration ×Time	3.34	3	1.11	1891	0.342

A Holm-Sidak test was performed to determine which colchicine concentrations were significantly different from each other with regards to the root length of the *E. urophylla* and *E. grandis* seedlings (Table 3.6). The results revealed that all concentrations were significantly ($p<0.05$) different from each other.

Table 3.6 Holm-Sidak test investigating the colchicine concentration effect on the root length of *E. urophylla* and *E. grandis* seedlings.

Colchicine concentration (%)	Mean Root length ± SD	Holm-Sidak test (p<0.05)*
Control	1.19 ± 0.52	A
0.01	1.06 ± 0.44	B
0.03	0.93 ± 0.43	C
0.05	0.78 ± 0.44	D

*Means with the same letter are non-significantly different.

A Holm-Sidak test was performed to determine which species and colchicine concentration interactions were significantly different from each other with regards to root length of *E. urophylla* and *E. grandis* seedlings (Table 3.7). The results revealed that *E. urophylla* control was significantly ($p<0.05$) different to all the other colchicine concentrations; and 0.01% and 0.03% colchicine treatments from *E. urophylla* had significantly ($p>0.05$) similar mean root lengths. The results also revealed that 0.03% and 0.05% treatments from *E. urophylla, E. grandis* control treatment, and 0.01% and 0.03% treatments from *E. grandis* were all significantly ($p>0.05$) similar. *E. grandis* 0.05% treatment was significantly ($p<0.05$) different from all the other treatments except for 0.03% from *E. grandis*.

Table 3.7 Holm-Sidak test investigating species and colchicine concentration interaction effect on the root length of *E. urophylla* and *E. grandis* seedlings.

Species	Colchicine concentration (%)	Mean Root length ± SD	Holm-Sidak test (p<0.05)*
E. urophylla	Control	1.42 ± 0.53	A
	0.01	1.18 ± 0.45	B
	0.03	1.06 ± 0.46	BC
	0.05	0.88 ± 0.47	CD
E. grandis	Control	0.93 ± 0.34	CD
	0.01	0.91 ± 0.37	CD
	0.03	0.79 ± 0.35	DE
	0.05	0.67 ± 0.37	E

*Means with the same letter are non-significantly different.

To investigate the effects of species, colchicine concentration and exposure time on the shoot length of the *E. urophylla* and *E. grandis* seedlings a Restricted Maximum Likelihood (REML) analysis was performed (Table 3.8). The REML analysis revealed that colchicine concentration ($p<0.001$), exposure time ($p<0.001$), the interaction between species and colchicine concentration ($p<0.001$) and the interaction between species and exposure time ($p<0.05$) had a significant effect on the shoot length of the *E. urophylla* and *E. grandis* seedlings.

Table 3.8 REML analysis on the shoot length of *E. urophylla* and *E. grandis* seedlings.

Source of variation	Wald statistic	n.d.f.	F value	d.d.f.	p value
Species	1.2	1	1.2	13	0.293
Colchicine concentration	399.32	3	133.11	1891	<0.001
Time	14.34	1	14.34	1891	<0.001
Species × Colchicine concentration	24.31	3	8.1	1891	<0.001
Species ×Time	7.54	1	7.54	1891	0.006
Colchicine concentration × Time	2.55	3	0.85	1891	0.467
Species × Colchicine concentration × Time	3.09	3	1.03	1891	0.379

A Holm-Sidak test was performed to determine which colchicine concentrations were significantly different from each other with regards to shoot length of *E. urophylla* and *E. grandis* seedlings (Table 3.9). The results revealed that all concentrations were significantly ($p<0.05$) different from each other with regards to shoot length of *E. urophylla* and *E. grandis* seedlings.

Table 3.9 Holm-Sidak test investigating the colchicine concentration effect on the shoot length of *E. urophylla* and *E. grandis* seedlings.

Colchicine concentration (%)	Mean Shoot Length ± SD	Holm-Sidak test ($p<0.05$)*
Control	1.35 ± 0.35	A
0.01	1.21 ± 0.35	B
0.03	1.05 ± 0.38	C
0.05	0.95 ± 0.41	D

*Means with the same letter are non-significantly different.

A Holm-Sidak test was performed as a *post hoc* test to determine which interactions between species and colchicine concentration were significantly different from each other with regards to shoot length of the seedlings (Table 3.10). The results revealed that the *E. urophylla* shoot length of the control treatment was significantly ($p<0.05$) different from all other treatments. *E. urophylla* 0.01% and 0.03% treatments as well as *E. grandis* control, 0.01% and 0.03% treatments were all significantly ($p>0.05$) similar with regards to shoot length. *E. urophylla* 0.03% and 0.05% treatments were significantly ($p>0.05$) similar to *E. grandis* 0.03% and 0.05% treatments with regards to shoot length.

Table 3.10 Holm-Sidak test investigating the species and concentration interaction effect on the shoot length of *E. urophylla* and *E. grandis* seedlings.

Species	Colchicine concentration (%)	Mean Shoot Length ± SD	Holm-Sidak test (p<0.05)*
E. urophylla	Control	1.45 ± 0.36	A
	0.01	1.23 ± 0.38	B
	0.03	1.07 ± 0.42	BCD
	0.05	0.99 ± 0.42	CD
E. grandis	Control	1.23 ± 0.30	B
	0.01	1.17 ± 0.31	BC
	0.03	1.03 ± 0.33	BCD
	0.05	0.91 ± 0.40	D

*Means with the same letter are non-significantly different.

A Holm-Sidak test was performed to determine which interaction between species and exposure times were significantly different from each other with regards to shoot length for *E. urophylla* and *E. grandis* (Table 3.11). The results revealed that the shoot length for the 24 h time exposure for *E. grandis* was significantly ($p<0.05$) different from all the other time exposures. The other time exposures (*E. grandis* 18 h, *E. urophylla* 18 h and *E. urophylla* 24 h) were all significantly ($p>0.05$) similar.

Table 3.11 Holm-Sidak test investigating the species and time exposure interaction effect on the shoot length of *E. urophylla* and *E. grandis* seedlings.

Species	Time (h)	Mean Shoot length ± SD	Holm-Sidak test (p<0.05)*
E. urophylla	18	1.20 ± 0.43	A
	24	1.18 ± 0.42	A
E. grandis	18	1.14 ± 0.35	A
	24	1.04 ± 0.36	B

*Means with the same letter are non-significantly different.

3.2.2 Phenotypic assessment

A phenotypic assessment was performed on the seedlings to investigate the effects of the induction procedure on the phenotype of the seedlings. The assessment was carried out by determining the survival of the seedlings as well as the number of atypical surviving seedlings in each treatment from each seedlot. In both procedures the number of replications was not included; instead all replications per treatment were measured and grouped together to indicate the colchicine effects in terms of phenotypic expression.

Seedling survival

The survival per sixteen seedlings of the eight seedlots for *E. urophylla* and seven seedlots for *E. grandis* seedlings were recorded two months after initiation of germination. The survival for each colchicine concentration in combination with a particular exposure time was calculated for each seedlot within each species (Table 3.12). It was also noted that particular seedlots displayed a greater susceptibility to the effects of an increase in colchicine concentration and exposure time compared with other seedlots. This was observed in *E. urophylla* seedlots U-125 and U-140, and *E. grandis* seedlots B4-415 and B4-873 that displayed the greatest variation in survival between treatments (within a seedlot) than other seedlots. Conversely, the seedlots U-123 of *E. urophylla* and B4-920 of *E. grandis* exhibited the highest survival with an increase in colchicine concentration and exposure time.

Table 3.12 Survival rates of different seedlots (out of a total of 16 seedlings per treatment per seedlot) for *E. urophylla* and *E. grandis.*

Seedlot	colchicine concentration (%) and exposure time (h)								Range in survival
	Control 18	Control 24	0.01 18	0.01 24	0.03 18	0.03 24	0.05 18	0.05 24	
E. urophylla									
U-116	12	9	10	8	13	10	13	3	10
U-123	11	10	4	4	4	8	5	9	7
U-124	13	16	12	9	10	4	7	6	12
U-125	16	14	10	7	2	2	3	3	14
U-135	16	16	14	7	9	8	5	5	11
U-138	15	12	11	8	8	3	5	4	12
U-139	11	14	13	8	7	10	3	2	12
U-140	15	16	15	15	4	8	2	6	14
E. grandis									
B4-54	11	13	7	11	5	4	2	6	11
B4-415	14	16	16	15	10	4	13	3	13
B4-553	12	14	13	12	11	5	10	4	10
B4-645	14	14	13	14	11	6	10	5	9
B4-689	13	16	14	15	15	11	12	6	10
B4-873	8	15	6	9	6	5	2	3	13
B4-920	15	16	15	15	15	14	12	10	6

A trend was evident when the seedlots were grouped according to treatment on a species level, the seedling survival decreased with an increase in colchicine concentration and exposure time (Table 3.13). Over and above a seedlot difference in survival observed in Table 3.12, a species diference was also noted. Overall *E. urophylla* displayed an 11 % lower survival rate than *E. grandis* (Table 3.13).

Table 3.13 Overall mean survival rates for the different treatments for *E. urophylla* and *E. grandis.*

Species	Colchicine concentration (%) and exposure time (h) (±SD)								Overall Survival
	0.00 18	0.00 24	0.01 18	0.01 24	0.03 18	0.03 24	0.05 18	0.05 24	
E. urophylla	13.63 ± 2.13	13.38 ± 2.77	11.13 ± 3.92	8.25 ± 2.38	7.13 ± 3.91	6.63 ± 4.22	6.63 ± 5.67	4.75 ± 2.76	52%
E. grandis	11.00 ± 4.97	14.86 ± 1.21	12.00 ± 3.40	13.00 ± 3.11	10.43 ± 3.64	7.14 ± 3.16	8.14 ± 4.21	5.43 ± 2.25	63%

A graphical illustration depicts the survival response of the seedlings after colchicine exposure (Figure 3.5).

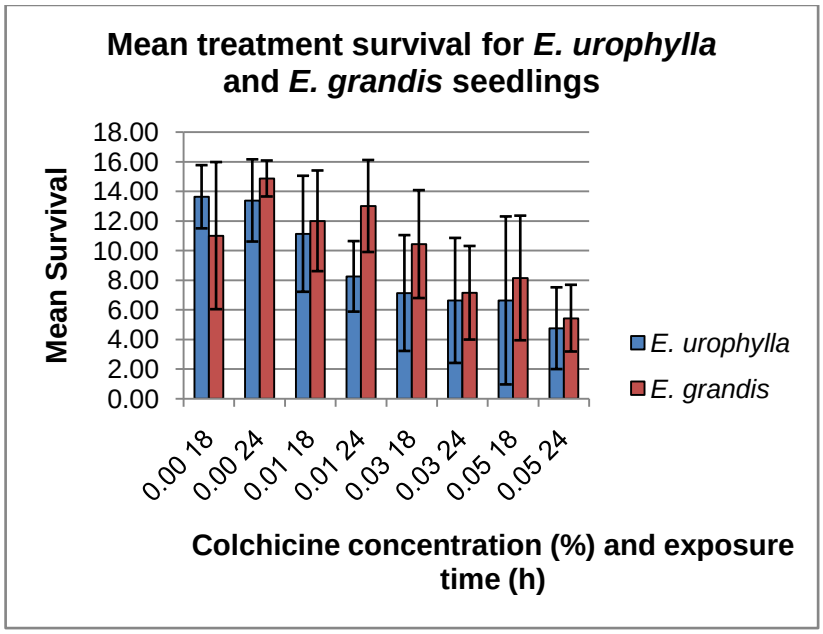

Figure 3.5 Comparison of mean survival of *E. urophylla* and *E. grandis*.

A generalized linear mixed model (GLMM) analysis was performed to investigate the effects of species, colchicine concentration and exposure time and their interactions on survival of *E. urophylla* and *E. grandis* seedlings (Table 3.14). Colchicine concentration ($p<0.001$) and the interaction between species, colchicine concentration and exposure time ($p<0.05$) had a significant effect on the survival of the *E. urophylla* and *E. grandis* seedlings.

Table 3.14 GLMM analysis on the survival of *E. urophylla* and *E. grandis* seedlings.

Source of variation	Wald statistic	n.d.f	F value	d.d.f	p value
Species	0.84	1	0.84	12.6	0.378
Colchicine concentration	82.27	3	27.42	90.8	<0.001
Time	3.22	1	3.22	90.6	0.076
Species × Colchicine concentration	7.64	3	2.55	90.9	0.061
Species × Time	0.5	1	0.5	90.6	0.481
Colchicine concentration × Time	7.32	3	2.45	90.6	0.069
Species × Colchicine concentration ×Time	10.51	3	3.5	90.6	0.019

A Holm-Sidak test was performed as a *post hoc* test to the GLMM to determine which colchicine concentrations were significantly different from each other with regards to seedling survival (Table 3.15). The results revealed that all the colchicine concentrations were significantly ($p<0.05$) different from each other except for 0.03% and 0.05% colchicine concentrations, which were significantly ($p>0.05$) similar, with regards to seedling survival in *E. urophylla* and *E. grandis* seedlings.

Table 3.15 Holm-Sidak test investigating the effect of colchicine concentration on survival of *E. urophylla* and *E. grandis* seedlings.

Colchicine concentration (%)	Mean Survival ± SD	Holm-Sidak test (p<0.05)*
Control	13.233 ± 3.202	A
0.01	11.000 ± 3.572	B
0.03	7.767 ± 3.839	CD
0.05	6.200 ± 3.925	D

*Means with the same letter are non-significantly different.

A Holm-Sidak test was performed to determine which species, colchicine concentration and exposure time interactions were significantly different from each other with regards to *E. urophylla* and *E. grandis* seedling survival (Table 3.16). The results follow the trend, where generally the lower concentrations had higher survival rates, which all tended to be significantly ($p>0.05$) similar. The seedlings treated with the higher concentrations and exposure times tended to have a lower survival rate, which were significantly ($p<0.05$) different to the survival rates of the lower concentrations. This is evident where, for example, the survival rate for both the controls in *E. urophylla* were significantly ($p<0.05$) different to the the survival rate of both *E. urophylla* 0.05% exposure times and 0.03% colchicine for 24 h exposure time.

Table 3.16 Holm-Sidak test investigating the effect of species, colchicine concentration and exposure time interactions on survival in *E. urophylla* and *E. grandis*.

Species	Colchicine concentration (%)	Exposure time (hours)	Mean Survival ± SD	Holm-Sidak test (p<0.05)*
E. urophylla	0.00	18	13.625 ± 2.134	AB
	0.00	24	13.375 ± 2.774	ABC
	0.01	18	11.125 ± 3.399	ABCD
	0.01	24	8.250 ± 3.105	BCD
	0.03	18	7.125 ± 3.643	CD
	0.03	24	6.625 ± 3.159	D
	0.05	18	6.625 ± 4.207	D
	0.05	24	4.75 ± 2.252	D
E. grandis	0.00	18	11.000 ± 4.970	ABCD
	0.00	24	14.857 ± 1.215	A
	0.01	18	12.000 ± 3.916	ABC
	0.01	24	13.000 ± 2.380	ABC
	0.03	18	10.429 ± 3.910	BCD
	0.03	24	7.143 ± 4.220	CD
	0.05	18	8.143 ± 5.670	BCD
	0.05	24	5.429 ± 2.760	D

*Means with the same letter are not significantly different.

Atypical seedlings

From germination up to the age of seven months it was noted that some seedlings displayed atypical growth. The seedlings displayed leaves that had irregular leaf margins, curly wrinkly leaves, little apical dominance and that were shorter than the control seedlings (Figure 3.6).

Figure 3.6 Seedlings grown from colchicine-induced seed displaying differences in phenotype. a. atypical phenotype and b. typical phenotype (control).

The seedlings (for both species) that exhibited atypical growth were predominantly found in the treatments exposed to higher colchicine concentrations (0.03% and 0.05%) for 24 h (Table 3.17 and 3.18). However, the treatments with the higher concentration and exposure times had a low survival rate and, therefore, the number of atypical seedlings represented in the table is not an accurate reflection of the true number had the survival rate been higher.

Table 3.17 Number of atypical seedlings identified from the surviving *E. urophylla* seedlings.

E. urophylla Seedlot	Colchicine concentration	18 hours		24 hours	
		Number seedling survival	Number (%*) atypical seedlings from the surviving seedlings	Number seedling survival	Number (%*) atypical seedlings from the surviving seedlings
U-116	0.00	12	0 (0.0)	9	0 (0.0)
	0.01	10	0 (0.0)	8	1 (12.5)
	0.03	13	3 (23.1)	10	2 (20.0)
	0.05	13	4 (30.8)	3	3 (100.0)
U-123	0.00	11	0 (0.0)	10	0 (0.0)
	0.01	4	3 (75.0)	4	0 (0.0)
	0.03	4	1 (25.0)	8	4 (50.0)
	0.05	5	2 (40.0)	9	2 (22.2)
U-124	0.00	13	0 (0.0)	16	0 (0.0)
	0.01	12	0 (0.0)	9	0 (0.0)
	0.03	10	0 (0.0)	4	1 (25.0)
	0.05	7	1(14.3)	6	3 (50.0)
U-125	0.00	16	0 (0.0)	14	0 (0.0)
	0.01	10	0 (0.0)	7	1 (14.3)
	0.03	2	1 (50.0)	2	2 (100.0)
	0.05	3	3 (100.0)	3	3 (100.0)
U-135	0.00	16	0 (0.0)	16	0 (0.0)
	0.01	14	0 (0.0)	7	0 (0.0)
	0.03	9	0 (0.0)	8	0 (0.0)
	0.05	5	0 (0.0)	5	1 (20.0)
U-138	0.00	15	0 (0.0)	12	0 (0.0)
	0.01	11	2 (18.9)	8	1 (12.5)
	0.03	8	4 (50.0)	3	0 (0.0)
	0.05	5	1 (20.0)	4	2 (50.0)
U-139	0.00	11	0 (0.0)	14	0 (0.0)
	0.01	13	1 (7.7)	8	1 (12.5)
	0.03	7	1 (14.3)	10	1 (12.5)
	0.05	3	0 (0.0)	2	2 (100.0)
U-140	0.00	15	0 (0.0)	16	0 (0.0)
	0.01	15	0 (0.0)	15	0 (0.0)
	0.03	4	1(25.0)	8	0 (0.0)
	0.05	2	1(50.0)	6	3 (50.0)

***Percentage of atypical seedlings calculated out of the number of surviving seedlings**

Table 3.18 Number of atypical seedlings identified from the surviving *E. grandis* seedlings.

E. grandis Seedlot	Colchicine concentration	18 hours		24 hours	
		Number seedling survival	Number (%*) atypical seedlings from the surviving seedlings	Number (%) survival	Number (%*) atypical seedlings from the surviving seedlings
B4-54	0.00	11	0 (0.0)	13	0 (0.0)
	0.01	7	0 (0.0)	11	0 (0.0)
	0.03	5	0 (0.0)	4	1 (25.0)
	0.05	2	1 (50.0)	6	1 (6.3)
B4-415	0.00	14	0 (0.0)	16	0 (0.0)
	0.01	16	0 (0.0)	15	0 (0.0)
	0.03	10	0 (0.0)	14	1 (7.1)
	0.05	13	1 (76.9)	13	1 (7.8)
B4-553	0.00	12	0 (0.0)	14	0 (0.0)
	0.01	13	0 (0.0)	12	0 (0.0)
	0.03	11	1 (9.1)	5	1 (20.0)
	0.05	10	2 (20.0)	4	1 (25.0)
B4-645	0.00	14	0 (0.0)	14	0 (0.0)
	0.01	13	0 (0.0)	14	0 (0.0)
	0.03	11	1 (9.1)	6	0 (0.0)
	0.05	10	1 (10.0)	5	0 (0.0)
B4-689	0.00	13	0 (0.0)	16	0 (0.0)
	0.01	15	0 (0.0)	15	0 (0.0)
	0.03	14	0 (0.0)	11	0 (0.0)
	0.05	12	1 (8.3)	6	1 (16.7)
B4-879	0.00	8	0 (0.0)	15	0 (0.0)
	0.01	6	0 (0.0)	9	2 (22.2)
	0.03	6	1 (16.7)	5	0 (0.0)
	0.05	2	0 (0.0)	3	0 (0.0)
B4-920	0.00	15	0 (0.0)	16	0 (0.0)
	0.01	15	0 (0.0)	15	0 (0.0)
	0.03	15	0 (0.0)	14	1 (7.1)
	0.05	12	1 (8.3)	10	2 (12.5)

***Percentage of atypical seedlings calculated out of the number of surviving seedlings**

The total number of atypical seedlings was calculated for each treatment for each species (Table 3.19). Generally, the higher the colchicine concentration and exposure time the higher the number of atypical seedlings found. Apart from *E. urophylla* exhibiting the lowest survival rate, *E. urophylla* also exhibited 75% more atypical seedlings than that of *E. grandis*.

Table 3.19 Total number of atypical seedlings found in each treatment for *E. urophylla* and *E. grandis*.

Species	Colchicine concentration (%) and exposure time (h)								
	0.00 18	0.00 24	0.01 18	0.01 24	0.03 18	0.03 24	0.05 18	0.05 24	Total number
E. urophylla	0	0	6	4	11	10	12	19	62
E. grandis	0	0	0	2	3	7	4	6	22

A generalized linear mixed model (GLMM) analysis was performed to investigate the effect of species, colchicine concentration, exposure time as well as the interaction between the fixed terms on the number of atypical *E. urophylla* and *E. grandis* seedlings (Table 3.20). Only species ($p<0.05$) and colchicine concentration ($p<0.001$) had a significant effect on the number of observed atypical *E. urophylla* and *E. grandis* seedlings.

Table 3.20 GLMM analysis on the number of atypical *E. urophylla* and *E. grandis* seedlings.

Source of variation	Wald statistic	n.d.f	F statistic	d.d.f	F pr
Species	5.5	1	5.5	14.4	0.034
Colchicine concentration	24.16	3	8.05	91.3	<0.001
Time	0.31	1	0.31	91.3	0.58
Species × Colchicine concentration	0.21	3	0.07	91.3	0.976
Species × Time	0.12	1	0.12	91.3	0.729
Colchicine concentration × Time	2.16	3	0.72	91.3	0.542
Species × Colchicine concentration × Time	1.55	3	0.52	91.3	0.672

A Holm-Sidak test was performed to determine which colchicine concentrations were significantly different with regards to the number of atypical *E. urophylla and E. urophylla* seedlings (Table 3.21). These results revealed that the control was significantly ($p<0.05$) different from the 0.03% and 0.05% colchicine concentrations, while the 0.01% colchicine concentration was only significantly ($p<0.05$) different from the 0.05% colchicine concentration with regards to the number of atypical *E. urophylla* and *E. grandis* seedlings observed.

Table 3.21 Holm-Sidak test investigating the effect of colchicine concentration on the number of atypical *E. urophylla* and *E. grandis* seedlings.

Colchicine concentration (%)	Mean number of atypical seedlings ± SD	Holm-Sidak test ($p<0.05$)*
0.00 (control)	0.000 ± 0.000	A
0.01	0.400 ± 0.770	AB
0.03	0.933 ± 0.112	BC
0.05	1.467 ± 1.074	C

*Means with the same letter are not significantly different.

3.3 INVESTIGATION 2: INDUCTION OF POLYPLOIDY IN AXILLARY BUDS

Polyploidy was induced into two *E. grandis* species clones and four *E. grandis* interspecific hybrid clones by treating axillary buds with different concentrations of colchicine (0.00%, 0.05%, 1.0% and 1.5%) for three consecutive days; 20 buds per concentration per clone. The number of bud sports (emerging new growth) that emerged from the treated axillary buds was quantified two months after induction (Figure 3.7). The response to the colchicine treatment was also quantified by measuring the length of the bud sports two months after induction. Compared with the phenotypic aberrations observed in the seedlings, the induced bud sports showed little variation in phenotype compared with that of the control bud sports. Figure 3.8 illustrates the time line of the induction process.

Figure 3.7 One of four G×U 082 clones with four developed bud sports.

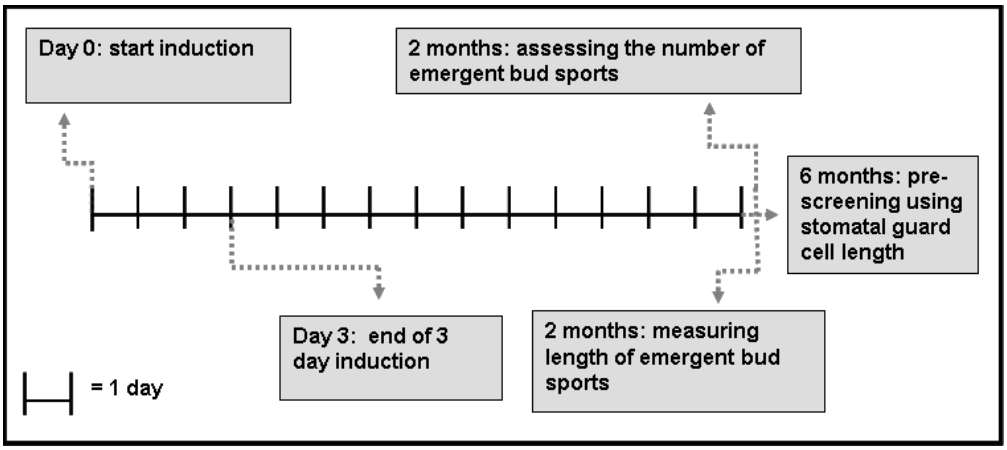

Figure 3.8 Timeline illustrating the induction phase and bud sport evaluation phases.

3.3.1 Growth of axillary buds

Comparing the number of emergent bud sports between treatments and within genotypes revealed no apparent pattern (Table 3.22). It would be expected that the colchicine would have, to an extent, a toxic effect on the meristematic cells, resulting in the lower number of emergent bud sports treated with the higher

colchicine conecntrations. However, only SGR 1266, SGR 1238 and GxU 082 had a lower number of emergent bud sports from the 1.50% colchicine treatment than the number from the control treatment, indicating a possible sensitivity to colchicine in only three out of the six genotypes. Comparing the number of emergent bud sports between treatments and for all genotypes (Table 3.22), reveals genotypes treated with 0.50% colchicine had the lowest mean number of bud sports (12.17). The genotypes treated with the two strongest colchicine concentrations (1.00% and 1.50%) had same mean number of emergent bud sports (13.17), however, more emergent bud sports than the 0.50% treatment.

Table 3.22 Number of emergent bud sports from the colchicine-treated axillary buds from the various genotypes.

Genotype	Colchicine concentration (%)			
	0.00	**0.50**	**1.00**	**1.50**
SGR 1266	13	12	11	14
SGR 1238	16	14	12	17
GxU 111	17	11	15	12
GxU 083	13	13	15	14
GxU 082	10	9	16	9
GxN 075	17	14	10	13
Mean ± SD	14.33 ± 2.80	12.17 ± 1.94	13.17 ± 2.48	13.17 ± 2.64

A generalized linear mixed model (GLMM) analysis was performed to investigate the effect of genotype on the number of emergent bud sports from the treated axillary buds (Table 3.23). Genotype had no significant effect ($p>0.05$) on the number of bud sports that emerged from the treated axillary buds.

Table 3.23 GLMM analysis on the effect of genotype on the number of emergent bud sports.

Source of variation	Wald statistic	n.d.f	F value	d.d.f	p value
Genotype	5.70	5	1.14	18.0	0.375

A generalized linear mixed model (GLMM) analysis was performed to investigate the effect of colchicine concentration on the number of emergent bud sports from the treated axillary buds (Table 3.24). Colchicine concentration had no significant effect ($p>0.05$) on the number of bud sports that emerged from the treated axillary buds.

Table 3.24 GLMM analysis on the effect of colchicine concentration on the number of emergent bud sports.

Source of variation	Wald statistic	n.d.f	F value	d.d.f	p value
Colchicine concentration	2.28	3	0.76	15.0	0.533

3.3.2 Growth of bud sports

The results of the mean bud sport length from the emergent bud sports revealed little trend, similar to that of the number of emergent bud sports (Table 3.25). It would be expected that the colchicine would have induced polyploidy in some meristematic cells, resulting in slower growth of the emergent bud sports compared to that of the control treatments. However, little variation in length of growth was identified between treatments and within genotype. Furthermore, some genotypes (SGR 1266, SGR 1238 and GxN 075) had longer bud sports grown from the colchicine treated axillary buds than the control treated axillary buds. Only GxU 111, GxU 083 and GxU 082 showed a decrease in the mean bud sport length in the colchicine treatments compared with the controls. When comparing the mean bud sport lengths between treatments and for all genotypes, the concentration of 1.00% colchicine resulted in the greatest retardation of growth.

Table 3.25 Mean bud sport length of the emergent bud sports from the various genotypes.

Genotype	Colchicine concentration (%)			
	0.00	**0.50**	**1.00**	**1.50**
SGR 1266	6.69 ± 2.24	6.69 ± 2.51	5.98 ± 1.99	7.29 ± 2.16
SGR 1238	7.30 ± 2.30	7.24 ± 2.72	7.03 ± 2.01	7.89 ± 1.94
GxU 111	7.18 ± 2.22	6.60 ± 2.66	6.75 ± 2.62	6.56 ± 2.24
GxU 083	7.57 ± 2.55	6.02 ± 2.68	6.50 ± 2.24	6.62 ± 2.47
GxU 082	8.28 ± 2.18	8.22 ± 2.04	7.35 ± 2.20	7.53 ± 1.95
GxN 075	6.36 ± 2.60	7.91 ± 2.03	6.38 ± 1.29	6.96 ± 1.96
Mean ± SD	7.28 ± 0.64	7.12 ± 0.84	6.66 ± 0.49	7.14 ± 0.53

A restricted maximum likelihood (REML) analysis was performed to investigate the effect of genotype and colchicine concentration on the length of the bud sports that emerged from the treated axillary buds (Table 3.26). The REML analysis revealed that genotype, colchicine concentration as well as the interaction between the two had no significant ($p>0.05$) effect on the length of the emergent bud sports.

Table 3.26 REML analysis on the length of the emergent bud sports.

Source of variation	Wald statistic	n.d.f	F value	d.d.f	p value
Genotype	8.92	5	1.78	293.0	0.116
Colchicine concentration	2.84	3	0.95	293.0	0.418
Genotype × Colchicine concentration	10.09	15	0.67	293.0	0.811

Generally, when the response to colchicine treatment on bud sport number and bud sport length were compared, it was found that, although not statistically significant, the number of bud sports were least at 0.5% colchicine treatment (Figure 3.9a), whereas 1.0% colchicine caused the greatest retardation in bud sport growth (Figure 3.10a).

CHAPTER 4

RESULTS: DETECTION OF POLYPLOIDY

4.1 INTRODUCTION

Both direct and indirect detection methods or a combination of the two are employed to screen for polyploidy in induced material in an array of different species. However, artificially induced polyploidy in *Eucalyptus* species has only been primarily detected by counting the chromosomes (Janaki *et al.*, 1969; Kampoor and Sharma, 1985). No attempt appears to have been made to indirectly assess polyploidy in *Eucalyptus*. Chromosome counts are difficult to perform in tree species including *Eucalyptus,* because of their small chromosomes (Hettasch, 1999; Beck *et al.*, 2005). As indirect methods are often easier than chromosome counting and allow for early identification of polyploidy, it was decided to develop such methods for the detection of polyploidy in *Eucalyptus*.

Many indirect methods exist which can be applied to different plant organs at various stages of the plant's development. Three indirect detection methods were selected; these methods included the measurement of stomatal guard cell length, stomatal frequency, and stomatal chloroplast frequency of the abaxial leaf surface. Stomatal guard cell length measurements were used as the initial screening for polyploidy. These stomatal measurements, however, are only indicative of the ploidy level of the epidermis, which originates from the first histogenic layer (Dolan and Poethig, 1998). It was, therefore, necessary to include a relatively rapid direct method used for polyploidy detection that indicates the ploidy level of all three histogenic layers. This method involved the quantification of DNA content using flow cytometry.

4.2 INVESTIGATION 3: DETECTION OF POLYPLOIDY IN SEEDLINGS

All seedlings, treated and controls (1024 *E. urophylla* and 896 *E. grandis*) were initially phenotypically screened after seven months. All seedlings with leaves that

were curly or that had irregular edges (atypical seedlings) were marked and labelled (discussed in Chapter 3). Three atypical seedlings from each treatment batch of 16 seedlings were selected and pre-screened for the presence of polyploidy by measuring the lengths of stomatal guard cells. For treatments with less than three atypical seedlings, the remaining seedlings were chosen randomly from the 16 seedlings of each treatment.

The true ploidy of the selected seedlings was then confirmed by quantifying the DNA content using flow cytometry. For comparison purposes, the stomatal frequencies and stomatal chloroplast frequencies were then determined and chloroplast arrangement in the guard cells noted. The cuttings of the reference material, the diploid hybrid (*E. grandis* x *E. camaldulensis*; SFX 104) and the tetraploid hybrid (*E. grandis* x *E. camaldulensis*; SFX 302), were also subjected to the different assessment procedures for comparison purposes, once it was confirmed that SFX 302 was a tetraploid using flow cytometry. Figure 4.1 illustrates the polyploidy detection procedure.

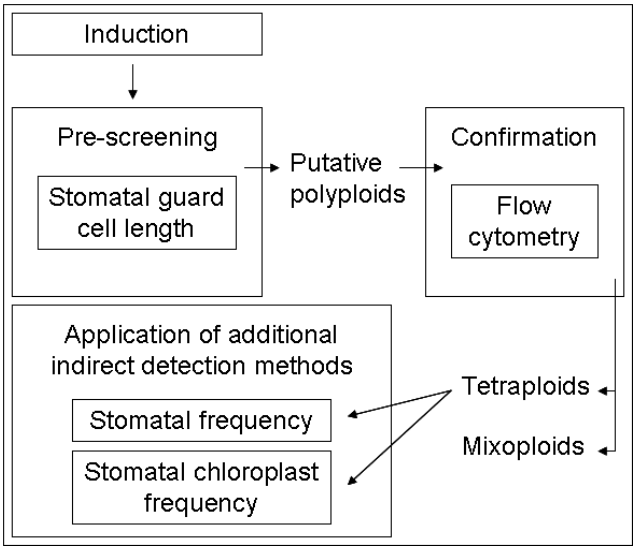

Figure 4.1 Flow diagram illustrating the polyploidy detection procedure in seedlings.

4.2.1 Pre-screening of seedlings for polyploidy

The measurement of stomatal guard cell length was used to pre-screen the selected *E. urophylla* and *E. grandis* seedlings, to identify putative polyploids. In general, the putative polyploids displayed a significant increase in stomatal guard cell length and a decreased stomatal frequency compared with the diploid control seedlings as shown in Figure 4.2. The sizes of normal epidermal cells of these putative polyploid seedlings also appeared to be substantially larger than that of the diploid controls.

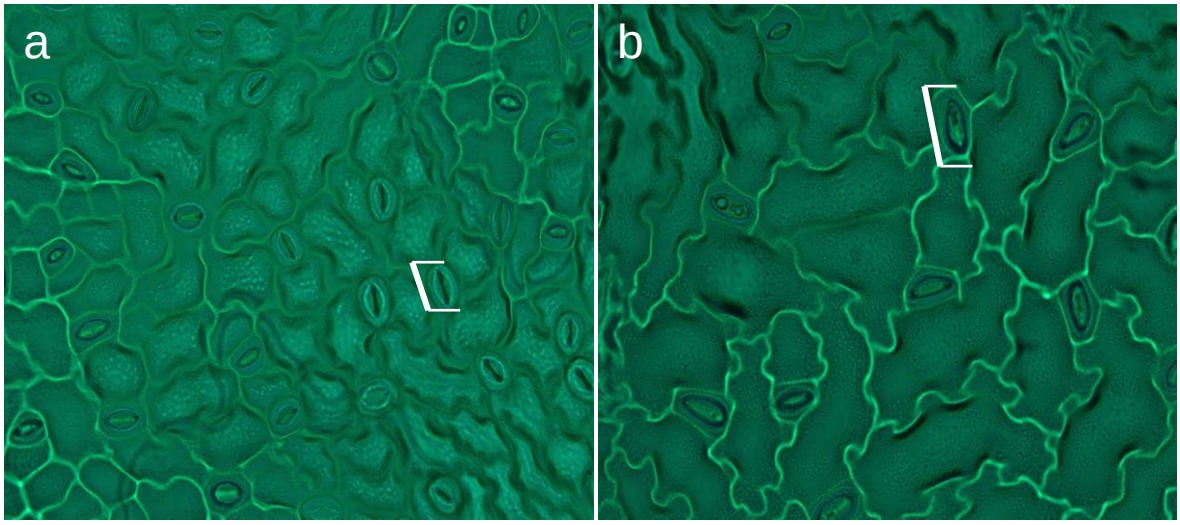

Figure 4.2 Stomatal length and frequency for a. diploid control and b. putative polyploid (0.05% colchicine concentration, 24 h exposure time) viewed with 40 x magnification.

Stomatal guard cell length measurements

The selected *E. urophylla* and *E. grandis* seedlings' stomatal guard cell lengths were measured. To identify seedlings with significantly different mean stomatal guard cell lengths from the controls, the mean stomatal guard cell lengths of all the treatments within one seedlot were grouped; after which an analysis of variance (ANOVA) was performed. In the case where the p value was smaller than 5%, a Bonferroni multiple comparison procedure was performed to identify which seedlings from which treatments were significantly ($p<0.05$) different in stomatal

guard cell length from the controls. These seedlings were labelled at putative polyploids.

Putative polyploids were detected in most of the *E. urophylla* and *E. grandis* seedlots. However, the number of putative polyploids differed between the different seedlots of the two species (Table 4.1). Putative polyploids were detected in six of the eight *E. urophylla* seedlots for all concentrations and exposure times, except for plants treated with 0.01% colchicine for 18 hours of exposure. Contrary to *E. urophylla*, *E. grandis* putative polyploid seedlings were observed in five of the seven seedlots for all three colchicine concentrations (0.01, 0.03, 0.05%), but only for 24 hour exposure. Only one putative polyploid was identified in each of the five *E. grandis* seedlots.

Table 4.1 Number of putative polyploid seedlings identified from a total of 9 pre-selected seedlings per seedlot in *E. urophylla* and *E. grandis*.

Species	Seedlots	colchicine concentration (%), time (h)	Number of identified putative polyploids
E. urophylla	U-116		0
	U-123	0.03 18 0.05 18 0.05 18 0.05 24	4
	U-124	0.05 24	1
	U-125	0.01 24 0.01 24 0.05 18 0.05 24	4
	U-135		0
	U-138	0.03 24 0.05 18 0.05 18 0.05 18	4
	U-139	0.03 18 0.03 24	2
	U-140	0.05 24 0.05 24	2
E. grandis	B4-54	0.05 24	1
	B4-415	0.05 24	1
	B4-553	0.03 24	1
	B4-645		0

B4-689		0
B4-873	0.01 24	1
B4-920	0.03 24	1

Mean stomatal guard cell lengths of the *E. urophylla* and *E. grandis* putative polyploids were compared with that of the diploid controls (Table 4.2). The overall mean stomatal guard cell length was approximately 30% higher for the putative polyploids than for the diploid controls for both species. The mean stomatal guard cell length of *E. urophylla* ranged from 20.88 µm to 21.71 µm in the diploid controls, while stomatal guard cell length ranged from 26.24 µm to 32.00 µm in the putative polyploids. In *E. grandis* the mean stomatal guard cell length per seedlot ranged from 20.34 µm to 21.89 µm in the diploid controls and in the putative polyploids from 24.36 µm to 35.34 µm. In both species the mean stomatal guard cell length per seedlot of the diploid controls did not overlap with the values found for the putative polyploids. However, the ranges within individuals did show some overlap between the diploids and polyploids.

When the mean stomatal guard cell lengths of the reference material was compared with that of the two species, it was found that the reference diploid had substantially shorter guard cells than the mean stomatal guard cell length for both species' controls. On the other hand, the mean stomatal guard cell length of the reference tetraploid was in the lower order of the mean stomatal guard cell frequency for the putative polyploids measured for both species. The mean stomatal guard cell length of the reference tetraploid was found to be approximately 35% longer than that of the reference diploid.

Table 4.2 Mean stomatal guard cell lengths (µm) of diploid controls and identified putative polyploids for *E. urophylla* and *E. grandis* seedlings, and reference material.

Species (ploidy)	Individual (µm)*	Mean individual length (µm)	Range of stomatal length within individual (µm)	Mean seedlot length (µm)	Range of stomatal length within seedlot (µm)	Mean ploidy length (µm)	Range of stomatal length within ploidy (µm)
E. urophylla (2x)	U-123 Control P1, P2, P3			21.71	26.47 - 17.13	21.02	28.10-15.92
	U-124 Control P1, P2, P3			21.07	25.31 - 16.86		
	U-125 Control P1, P2, P3			20.99	25.54 - 15.92		
	U-138 Control P1, P2, P3			21.20	28.10 - 16.65		
	U-139 Control P1, P2, P3			20.88	26.12 - 17.28		
	U-140 Control P1, P2, P3			21.26	26.00 - 17.38		
E. urophylla ('4x')	U-123 0.03 18 P1	30.72	25.63 – 36.26	30.027	36.26-20.16	29.56	43.24-18.43
	U-123 0.05 18 P2	28.81	20.16 –33.87				
	U-123 0.05 18 P3	30.30	25.50 – 34.41				
	U-123 0.05 24 P2	30.27	24.53 – 34.64				
	U-124 0.05 24 P2	32.00	26.98 – 37.55	32.00	26.98 – 37.55		
	U-125 0.01 24 P1	29.15	24.26 – 34.89				
	U-125 0.01 24 P2	28.14	23.17 – 39.64	29.56	39.64-23.17		
	U-125 0.05 18 P2	31.53	27.47 – 37.40				
	U-125 0.05 24 P2	29.42	23.85 – 34.35				
	U-138 0.03 24 P1	27.88	24.69 – 32.65				
	U-138 0.05 18 P1	31.21	18.43 – 37.86	31.37	43.24-18.43		
	U-138 0.05 18 P2	36.25	29.59 – 43.24				
	U-138 0.05 18 P3	30.13	25.64 – 36.27				
	U-139 0.03 18 P1	32.71	28.61 – 38.04	29.91	38.04-24.09		
	U-139 0.03 24 P1	27.10	24.09 – 30.48				
	U-140 0.05 24 P1	27.87	23.51 – 36.33	26.24	36.33-21.88		
	U-140 0.05 24 P3	24.60	21.88 – 30.13				

*Naming of individual for example U-123 0.03 18 P1: U-123 = seedlot number; 0.03 = colchicine concentration; 18 = exposure time in hours and P1 = plant number.
2x denotes diploid; 4x denotes tetraploid and '4x' denotes putative tetraploid.

Table 4.2 (cont.) **Mean stomatal guard cell lengths (µm) of diploid controls and identified putative polyploids for *E. urophylla* and *E. grandis* seedlings, and reference material.**

Species (ploidy)	Individual (µm)*	Mean individual length (µm)	Range of stomatal length within individual (µm)	Mean seedlot length (µm)	Range of stomatal length within seedlot (µm)	Mean ploidy length (µm)	Range of stomatal length within ploidy (µm)
E. grandis (2x)	B4-54 Control P1, P2, P3			20.52	25.18 -16.65		
	B4-415 Control P1, P2, P3			21.89	28.31 -16.52		
	B4-553 Control P1, P2, P3			20.34	25.31 -15.19	21.04	28.31 -15.19
	B4-873 Control P1, P2, P3			20.71	24.40 -16.65		
	B4-920 Control P1, P2, P3			21.75	25.04 -18.36		
E. grandis ('4x')	B4-54 0.05 24 P1	30.30	35.22 – 25.47	30.30	35.22 - 25.47		
	B4-415 0.05 24 P1	35.34	40.89 – 24.51	35.34	40.89 - 24.51		
	B4-553 0.03 24 P1	26.80	30.25 – 21.26	26.80	30.25 - 21.26	29.01	40.89 -20.51
	B4-873 0.01 24 P3	28.25	33.43 – 24.19	28.25	33.43 - 24.19		
	B4-920 0.03 24 P1	24.36	30.09 – 20.51	24.36	30.09 - 20.51		
Reference (2x)	SFX 104					17.54	13.63 – 21.58
Reference (4x)	SFX 302					26.57	19.48 – 34.99

*Naming of individual for example B4-54 0.05 24 P1: B4-54= seedlot number; 0.05 = colchicine concentration; 24 = exposure time in hours and P1 = plant number.
2x denotes diploid; 4x denotes tetraploid and '4x' denotes putative tetraploid.

Figure 4.3 shows the mean stomatal guard cell lengths of the diploid controls and putative polyploids of *E. urophylla*, *E. grandis* and the reference diploid and tetraploid. The putative polyploids clearly had longer stomatal guard cells than the diploids.

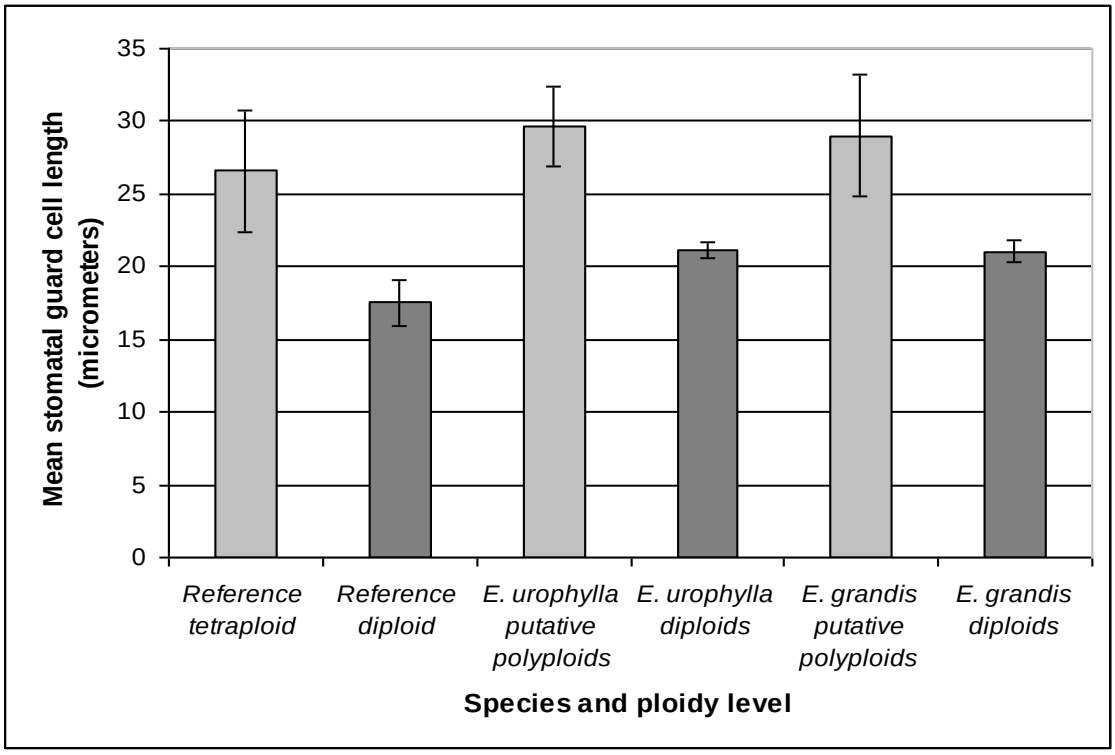

Figure 4.3 **Mean stomatal guard cell length of the reference tetraploid, reference diploid, *E. urophylla* and *E. grandis* putative polyploids and corresponding diploids.**

4.2.2 Confirmation of polyploidy in seedlings and reference material

The ploidy level of the putative polyploids identified using stomatal guard cell length measurements were confirmed by quantifying the DNA content using flow cytometry. It was expected that the amount of DNA of a putative polyploid would be double that of the diploid from which it was derived. Flow cytometry was also used to verify that the reference tetraploid was indeed polyploid.

Flow cytometry

The DNA content depicted by the histograms generated by the flow cytometer confirmed that the reference polyploid was a tetraploid (Figure 4.4). This is illustrated by the DNA peak of the tetraploid being at its highest point in channel 415 (Figure 4.4a), whereas in the reference diploid the DNA peak was in channel 200 (Figure 4.4b). This indicates that the tetraploid's DNA content was approximately double that of the reference diploid.

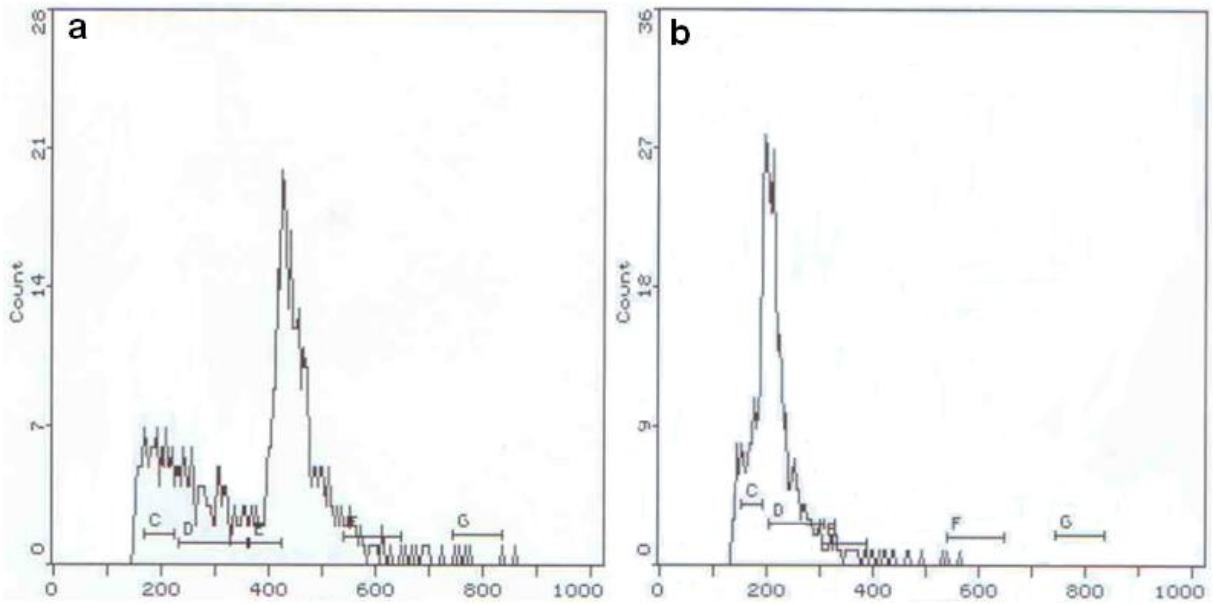

Figure 4.4 Histograms displaying the relative DNA content of leaf nuclei for a. reference tetraploid and b. reference diploid.

The ploidy of putative polyploid seedlings identified from stomatal length measurements was investigated by flow cytometry. Figure 4.5a demonstrates the tetraploid condition of *E. urophylla* U-123 seedling germinated in 0.03% colchicine for 18 h. The tetraploid displayed a DNA peak at approximately channel 500, while the histogram of the U-123 diploid control displayed a DNA peak at approximately channel 250 (Figure 4.5b). Similar to *E. urophylla*, seedling B4-415 of *E. grandis* germinated in 0.05% colchicine for 24 h was also confirmed to be tetraploid

(Figure 4.5c). The tetraploid peaked at channel 450; while the B4-415 diploid control peaked at channel 225 (Figure 4.5d).

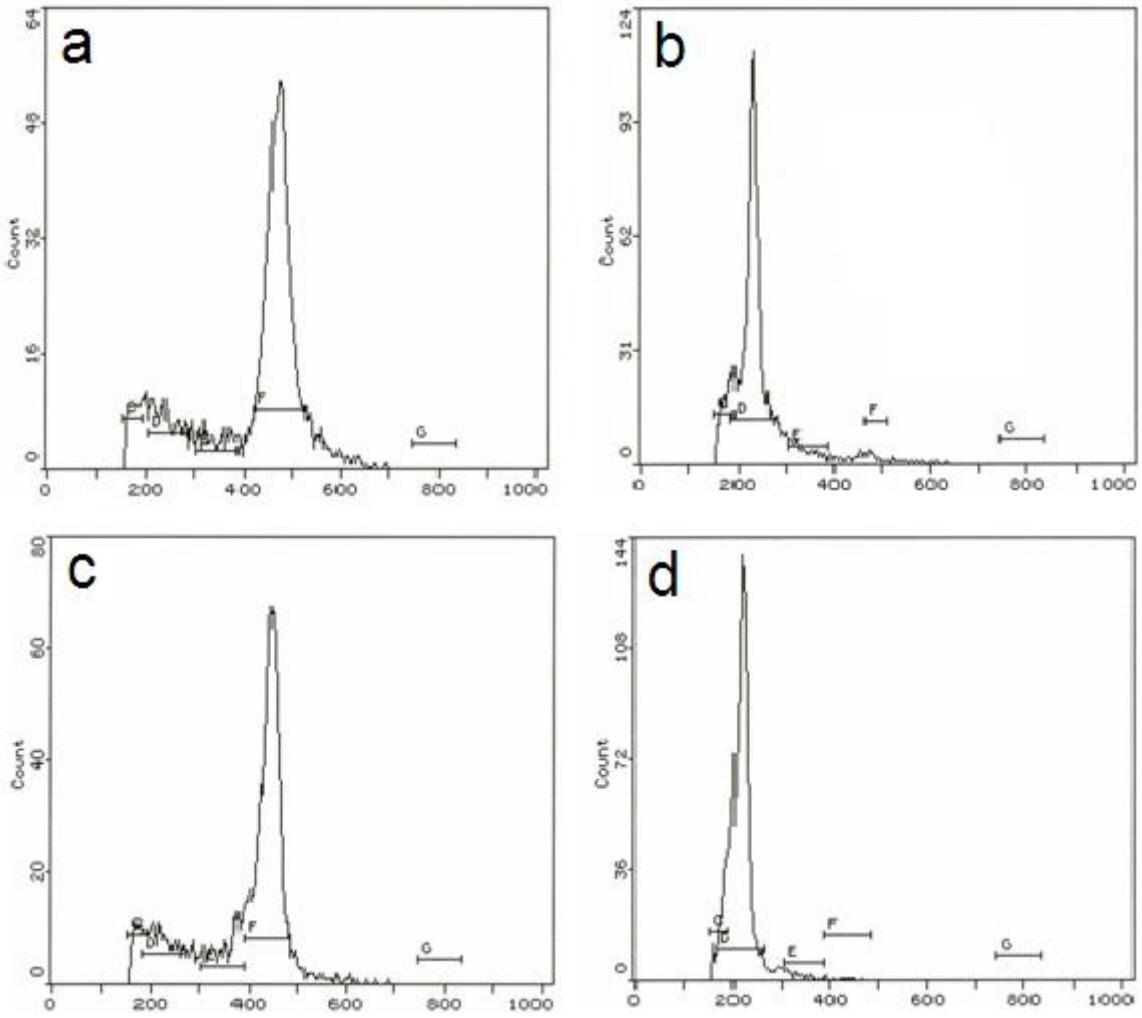

Figure 4.5 Flow cytometry histograms displaying relative DNA content of leaf nuclei for a. *E. urophylla* tetraploid, b. *E. urophylla* diploid control, c. *E. grandis* tetraploid and d. *E. grandis* diploid control.

A number of the putative polyploids turned out to be mixoploids, where regions of both the sample leaves were diploid in nature and other regions tetraploid in nature. Figures 4.6a and b demonstrate the dual nature of the histograms for an *E. urophylla* seedling U-123 germinated in 0.05% colchicine for 18 h and for an *E. grandis* seedling B4-553 germinated in 0.03% colchicine for 24 h. These histograms show two peaks; diploid peak in channel 225 and tetraploid peak in channel 450, confirming that both cell types were present.

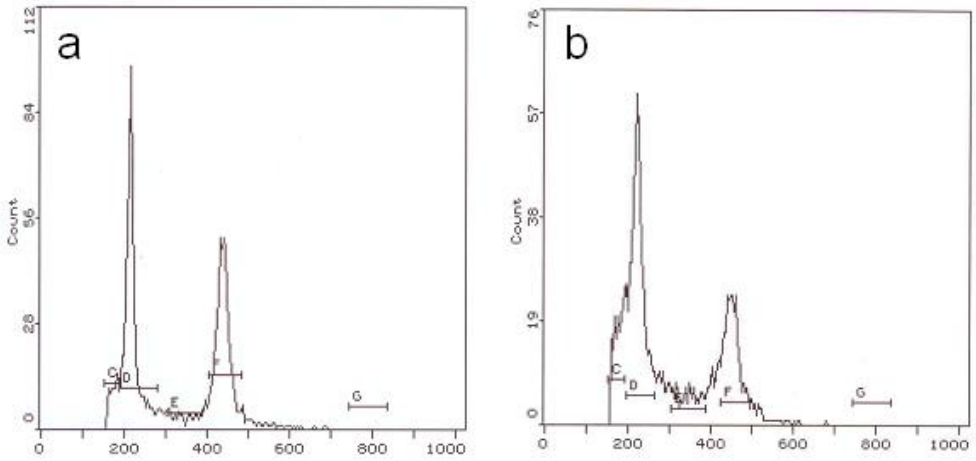

Figure 4.6 Histograms of a. *E. urophylla,* and b. *E. grandis* mixoploids.

Of the 17 *E. urophylla* putative polyploid seedlings six were tetraploid and of the five *E. grandis* putative polyploid seedlings only one was tetraploid (Table 4.3). The remainder of the putative polyploids from *E. urophylla* and *E. grandis* were mixoploids according to the flow cytometry analysis.

Table 4.3 Putative polyploid confirmation results.

Species	Seedlot	Colchicine concentration (%)	Colchicine exposure (h)	Seedling	Flow cytometry results
E. urophylla	U-123	0.05	24	P2	4x = Tetraploid
	U-123	0.05	18	P2	2x + 4x = Mixoploid
	U-123	0.05	18	P3	2x + 4x = Mixoploid
	U-123	0.03	18	P1	4x = Tetraploid
	U-124	0.05	24	P2	2x + 4x = Mixoploid
	U-125	0.01	24	P2	4x = Tetraploid
	U-125	0.05	18	P2	4x = Tetraploid
	U-125	0.01	24	P1	2x + 4x = Mixoploid
	U-125	0.05	24	P2	4x = Tetraploid
	U-138	0.05	18	P3	2x + 4x = Mixoploid
	U-138	0.05	18	P2	2x + 4x = Mixoploid
	U-138	0.05	18	P1	2x + 4x = Mixoploid
	U-138	0.03	24	P1	2x + 4x = Mixoploid
	U-139	0.03	18	P1	4x = Tetraploid
	U-139	0.03	24	P1	2x + 4x = Mixoploid
	U-140	0.05	24	P2	2x + 4x = Mixoploid
	U-140	0.05	24	P1	2x + 4x = Mixoploid
E. grandis	B4-54	0.05	24	P1	2x + 4x = Mixoploid
	B4-415	0.05	24	P1	4x = Tetraploid
	B4-553	0.03	24	P1	2x + 4x = Mixoploid
	B4-873	0.01	24	P3	2x + 4x = Mixoploid
	B4-920	0.03	24	P1	2x + 4x = Mixoploid

The conversion rate of diploidy to polyploidy was calculated for both *E. urophylla* and *E. grandis* following the identification of true tetraploids by flow cytometry by dividing the number of true tetraploids by the number of sampled seedlings (Table 4.4). *E. urophylla* seedlings exhibited approximately 80% higher tetraploid conversion than that of *E. grandis*.

Table 4.4 Tetraploid conversion rates for *E. urophylla* and *E. grandis* induced seedlings.

Species	Number treated	Number sampled	Number of putative polyploids*	Number of mixoploids**	Number of tetraploids***	Tetraploid conversion rate****
E. urophylla	768 (excl controls)	144 (excl controls)	17	11	6	4.2%
E. grandis	672 (excl controls)	126 (excl controls)	5	4	1	0.8%

*Number of putative polyploids detected from sample
**Number of mixoploids detected from the putative polyploids
***Number of tetraploids detected from the putative polyploids
****The percentage of tetraploids identified from the number of seedlings sampled

The tetraploidy conversion rate was then calculated for specific treatments to determine the treatments that were most successful in inducing tetraploidy in this experiment (Table 4.5). For *E. urophylla*, the treatments 0.03% 18 h and 0.05% 24 h exhibited the highest conversion rate of 8.4%. In *E. grandis*, only one tetraploid was detected, in the 0.05% 24 h treatment and therefore exhibited the highest conversion rate of 4.2%.

Table 4.5 Tetraploid conversion rate for the various colchicine treatments for *E. urophylla* and *E. grandis* seedlings.

Treatment	Number treated	Number sampled	Number of putative polyploids*	Number of mixoploids**	Number of tetraploids***	Tetraploid conversion rate****
E. urophylla						
0.01 18	128	24	0	0	0	0.0
0.03 18	128	24	2	0	2	8.4
0.05 18	128	24	6	5	1	4.2
0.01 24	128	24	2	1	1	4.2
0.03 24	128	24	2	2	0	0.0
0.05 24	128	24	5	3	2	8.4
E. grandis						
0.01 18	112	21	0	0	0	0.0
0.03 18	112	21	0	0	0	0.0
0.05 18	112	21	0	0	0	0.0
0.01 24	112	21	1	1	0	0.0
0.03 24	112	21	2	2	0	0.0
0.05 24	112	21	2	1	1	4.2

*Number of putative polyploids detected from sample
**Number of mixoploids detected from the putative polyploids
***Number of tetraploids detected from the putative polyploids
****The percentage of tetraploids identified from the number of seedlings sampled

4.2.3 The application of additional detection methods

Two additional detection methods were applied to the newly identified tetraploids to determine if these methods can be suitably used for polyploidy detection in *Eucalyptus*. These methods were stomatal frequency and stomatal chloroplast frequency and arrangement.

Stomatal frequency

The stomatal frequencies on the abaxial surface of the leaves of seven confirmed tetraploids and of the reference tetraploid, identified from flow cytometry, were compared with that of their corresponding diploids. Generally, the tetraploids

exhibited a decrease in the number of stomata when compared with the diploids (Figure 4.2).

To investigate whether stomatal frequency could be used as an accurate screening method for polyploidy detection, Mann-Whitney u-tests were applied to the tetraploids and their corresponding diploids to determine if their mean stomatal frequencies were significantly different (Table 4.6). The Mann-Whitney u-tests revealed that all the tetraploids were significantly ($p<0.001$) different from their corresponding diploids. Stomatal frequency is therefore an accurate screening method for polyploidy detection in *Eucalyptus.*

Table 4.6 Mann-Whitney u-tests determining the statistical difference in stomatal frequency between the tetraploids and their corresponding diploids.

Tetraploid			Diploid			U-test
Treatment	**Frequency**	**n**	**Treatment**	**Frequency**	**n**	**result**
U-123 0.03% 18 h	20.50 ± 1.73	12	U-123 control	57.04 ± 9.92	36	p<0.001
U-123 0.05% 24 h	24.25 ± 2.83	12	U-123 control	56.92 ± 9.72	36	p<0.001
U-125 0.01% 24 h	29.00 ± 5.59	12	U-125 control	64.79 ± 7.58	36	p<0.001
U-125 0.05% 18 h	21.83 ± 2.17	12	U-125 control	64.79 ± 7.58	36	p<0.001
U-125 0.05% 24 h	32.58 ± 3.80	12	U-125 control	64.79 ± 7.58	36	p<0.001
U-139 0.03% 18 h	17.58 ± 2.47	12	U-139 control	57.92 ± 5.69	36	p<0.001
B4-415 0.05% 24 h	25.92 ± 2.35	12	B4-415 control	77.25 ± 4.61	36	p<0.001
SFX 302	43.29 ± 7.80	48	SFX 104	51.90 ± 7.82	48	p<0.001

Stomatal chloroplast frequency and arrangement in seedlings

The frequency of the guard cell chloroplasts of four tetraploids, identified from flow cytometry as well as the reference hybrid tetraploid were compared with that of their corresponding diploids. Generally, the tetraploids had more chloroplasts in their guard cells than the diploids (Figure 4.7). Also, chloroplast arrangement differed (Figure 4.7). The greater number of chloroplasts in the tetraploid tended to be dispersed throughout a cell, while the smaller number of chloroplasts of a diploid tended to collect at the tips of the kidney shaped guard cells.

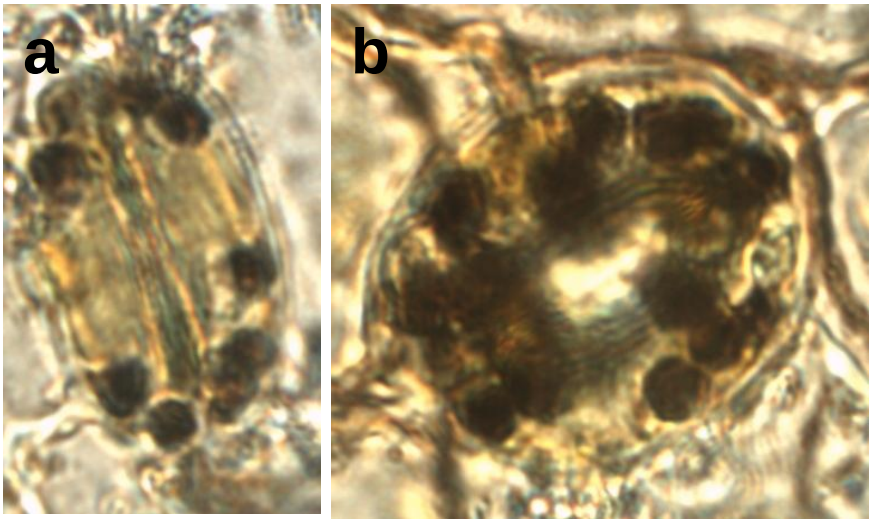

Figure 4.7 Chloroplast arrangements in stomatal guard cells. a. Polarised arrangement in a diploid and b. Dispersed arrangement in a tetraploid (viewed with 100 x magnification).

Mann-Whitney u-tests were applied to the tetraploids and their corresponding diploids to determine if their mean stomatal chloroplast frequencies were significantly different (Table 4.7). The Mann-Whitney u-tests revealed that the stomatal chloroplast frequencies of all the tetraploids were significantly ($p<0.001$) different from their corresponding diploids, indicating that this character is an accurate screening method for polyploidy detection in *Eucalyptus.*

Table 4.7 Man-Whitney u-tests determining the statistical difference in stomatal chloroplast frequency between the tetraploids and their corresponding diploids.

Tetraploids			Diploids			U-test
Treatment	Frequency	n	Treatment	Frequency	n	result
U-123 0.03% 18 h	19.00 ± 1.97	34	U-123 control	11.59 ± 1.39	34	p<0.001
U-125 0.05% 24 h	18.50 ± 2.14	34	U-125 control	11.88 ± 1.20	34	p<0.001
U-139 0.03% 18 h	18.74 ± 1.64	34	U-139 control	12.15 ± 1.13	34	p<0.001
B4-415 0.05% 24 h	19.77 ± 2.32	34	B4-415 control	10.77 ± 1.44	34	p<0.001
SFX 302	23.08 ± 3.51	48	SFX 104	13.06 ± 1.31	48	p<0.001

4.2.4 Comparison of phenotypic characteristics of confirmed tetraploid and mixoploid seedlings.

The morphology of the tetraploid and mixoploid seedlings were assessed to identify phenotypic traits that have the potential to be used for preliminary recognition of successfully induced polyploidy. The colchicine-induced mixoploid and tetraploid seedlings displayed distinct morphological differences when compared with control seedlings (Figure 4.8). These morphological differences were, however, inconsistent and included wrinkly leaves and jagged leaf margins, which resulted in unsymmetrical leaves. Other recognisable differences were lack of apical dominance and differences in leaf colour intensity. The leaves of the induced tetraploids were darker than those of the diploid controls of the same age, probably due to the increased chloroplast number, which is probably accompanied by an increase in the chlorophyll concentration. In some instances the tetraploids and mixoploids had altered leaf indices, exhibiting leaves which were larger and often thinner.

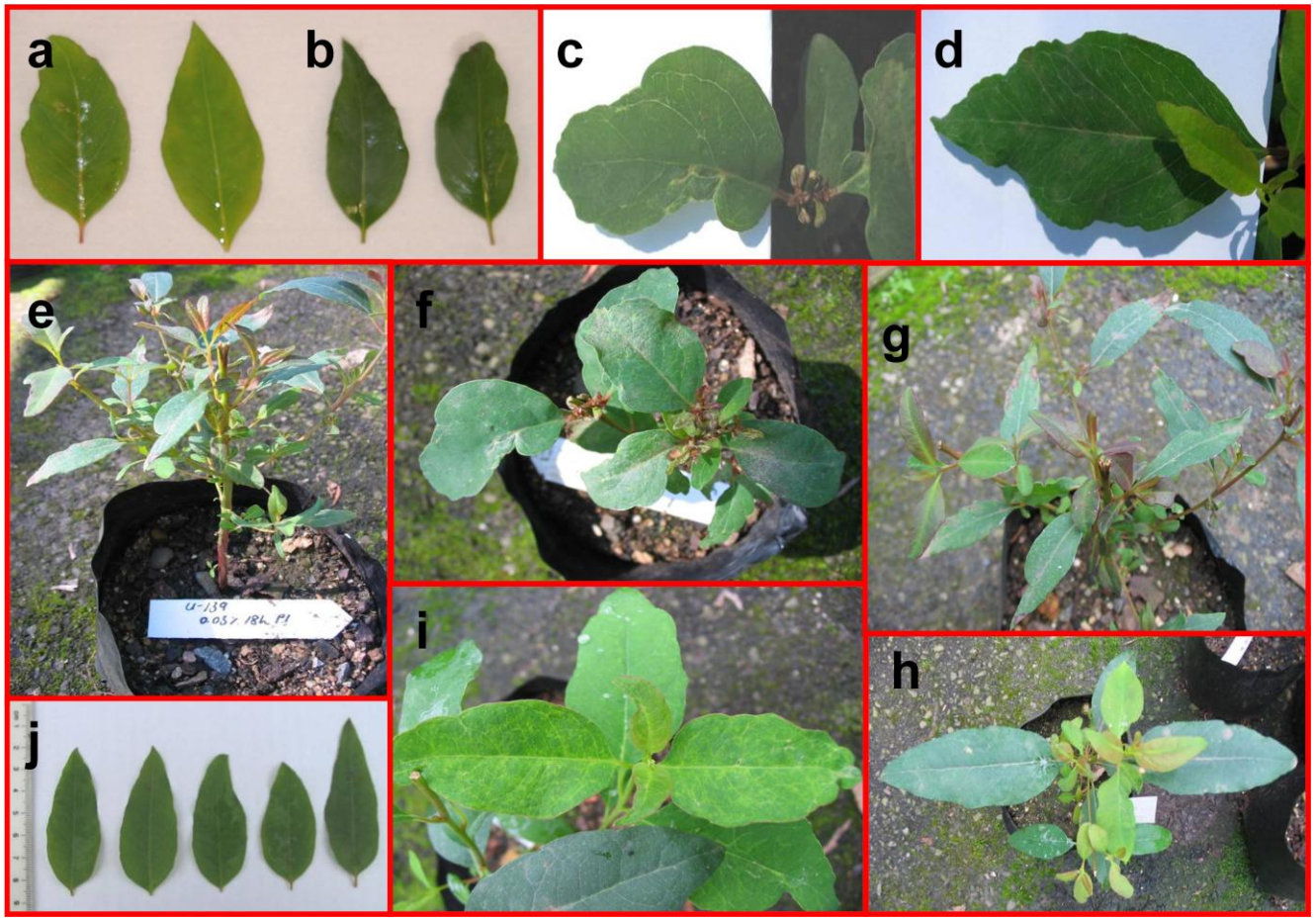

Figure 4.8 **Alteration in leaf morphology following the induction of polyploidy in seedlings. a. Leaf colouring for diploid control seedlings. b. Leaf colouring of polyploidy seedlings. c. Unsymmetrical leaf margins in tetraploids seedlings. d. Wavy leaf margins in tetraploid seedlings. e. High response to pruning with initial lack of apical dominance. f. Unsymmetrical leaf margins in tetraploid seedlings. g. Thinner leaves in some tetraploid seedlings. h. Larger leaves in a mixoploid seedling. i. Mottled green colouring for leaves of a mixoploid seedling. j. Consistent leaf symmetry size and colouring of leaves from diploid control seedlings.**

4.2 INVESTIGATION 3: DETECTION OF POLYPLOIDY IN BUD SPORTS

In the case of axillary induced polyploidy, the treated bud sports did not display any morphological changes that could distinguish them from the bud sport controls. Therefore, six bud sports from the induced axillary buds were randomly selected from a total of 20 bud sports per treatment per genotype and three bud sports from the control treatments. The selected bud sports were then pre-screened for the presence of polyploidy using stomatal guard cell length measurements and polyploidy confirmed in the putative polyploids (bud sports with significantly different stomatal guard cell length measurements from the controls) by quantifying DNA content using flow cytometry (Figure 4.9).

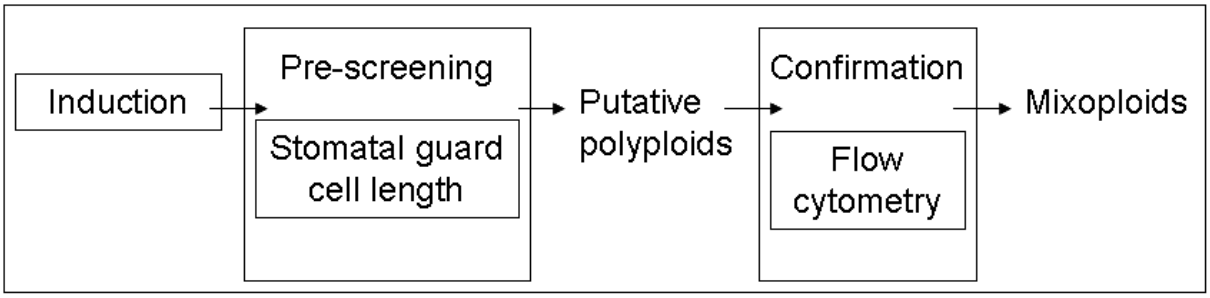

Figure 4.9 Flow diagram illustrating the polyploidy detection procedure in bud sports.

4.3.1 Pre-screening of bud sports for polyploidy

The measurement of stomatal guard cell length was used to pre-screen the selected bud sports. Generally, the putative polyploids displayed regions of the bud sport leaves that differed significantly in stomatal size. Some regions had larger stomata, while others had smaller stomata (Figure 4.10). These regions of enlarged stomata varied in area, from smaller regions containing only a few stomata, as little as five, to regions with the size of several fields of view.

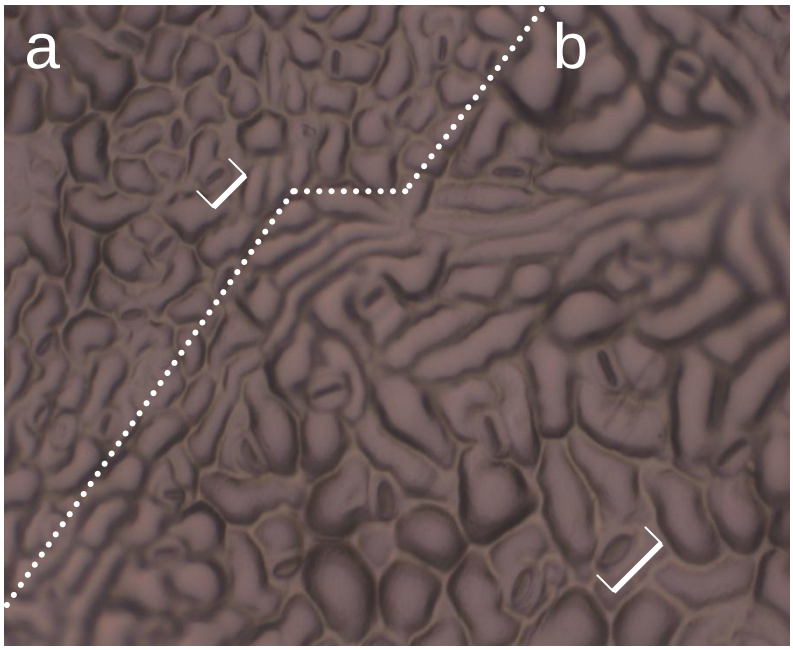

Figure 4.10 Leaf of mixoploid bud sport with, a. an area with smaller diploid-sized stomata, and b. an area with larger stomata viewed with 40× magnification.

Stomatal guard cell length

Bud sports grown from colchicine treated axillary buds were pre-screened by measuring stomatal lengths. Bud sports generated from colchicine treated axillary buds were preliminarily classified as putative polyploids when their leaves contained regions of both larger and smaller stomata. To classify the bud sports as putative polyploids statistically, the mean stomatal guard cell lengths of all the treatments within one genotype were grouped together; after which an analysis of variance (ANOVA) was performed per genotype. Where the p value was less than 5%, a Bonferroni correction was performed to identify which bud sports differed significantly from the control. The bud sports that had mean stomatal guard cell lengths that differed significantly from the controls were labelled as putative polyploids.

The most putative polyploids were detected in SGR 1238 and G×U 082 (Table 4.8). Putative polyploids were identified from all three colchicine concentrations;

however, the most putative polyploids were identified from the 1% colchicine concentration.

Table 4.8 Mean stomatal length (µm) of the putative polyploids and corresponding control bud sports.

Genotype	Colchicine concentration	Mean stomatal guard cell length ± SD
SGR 1266	1.0%	27.34 ± 5.63
SGR 1266	1.0%	24.29 ± 2.33
SGR 1266	0.0%	21.45 ± 0.27
SGR 1238	1.0%	26.98 ± 5.27
SGR 1238	1.5%	26.05 ± 4.80
SGR 1238	1.5%	27.07 ± 4.91
SGR 1238	1.5%	25.54 ± 4.33
SGR 1238	0.0%	20.49 ± 0.69
GxN 075	1.0%	26.57 ± 5.16
GxN 075	0.0%	21.33 ± 0.44
GxU 082	0.5%	23.30 ± 3.72
GxU 082	1.0%	24.76 ± 2.62
GxU 082	1.0%	26.38 ± 3.74
GxU 082	1.0%	25.16 ± 2.50
GxU 082	1.0%	25.41 ± 3.26
GxU 082	1.5%	25.61 ± 3.17
GxU 082	1.5%	24.70 ± 2.13
GxU 082	1.5%	24.52 ± 2.03
GxU 082	0.0%	20.29 ± 1.04
GxU 083	0.5%	24.03 ± 3.81
GxU 083	1.0%	24.40 ± 2.23
GxU 083	0.0%	20.71 ± 0.08
GxU 111	1.0%	24.60 ± 2.01
GxU 111	0.0%	20.07 ± 1.43

4.3.2 Confirmation of polyploidy in bud sports using flow cytometry

The DNA content of putative polyploids identified from stomatal length measurements was subsequently quantified using flow cytometry. The flow

cytometry results revealed that only a few bud sports were mixoploids and the remaining putative polyploid bud sports were diploids that had possibly reverted from mixoploids (Table 4.9).

Table 4.9 Flow cytometry results on the ploidy level of putative polyploidy bud sports.

Genotype	Treatment	Flow cytometry results
SGR 1266	1.0%	2x + 4x = Mixoploid
SGR 1266	1.0%	2x = Diploid
SGR 1238	1.0%	2x + 4x = Mixoploid
SGR 1238	1.5%	2x = Diploid
SGR 1238	1.5%	2x = Diploid
SGR 1238	1.5%	2x + 4x = Mixoploid
GxN 075	1.0%	2x + 4x = Mixoploid
GxU 082	0.5%	2x = Diploid
GxU 082	1.0%	2x = Diploid
GxU 082	1.0%	2x + 4x = Mixoploid
GxU 082	1.0%	2x = Diploid
GxU 082	1.0%	2x = Diploid
GxU 082	1.5%	2x = Diploid
GxU 082	1.5%	2x = Diploid
GxU 082	1.5%	2x = Diploid
GxU 083	0.5%	2x + 4x = Mixoploid
GxU 083	1.0%	2x = Diploid
GxU 111	1.0%	2x + 4x = Mixoploid

The histogram in Figure 4.11 depicts two different cell types isolated from a mixoploid bud sport that resulted from an axillary bud treated with 1.0% colchicine from an *E. grandis* species clone, SGR 1266. The mixoploid displayed two peaks, one at channel 200 and the other at channel 400, indicating that the leaf contains two different cell types (Figure 4.11a). The DNA peaked at channel 200 for the diploid control of SGR 1266 (Figure 4.11b).

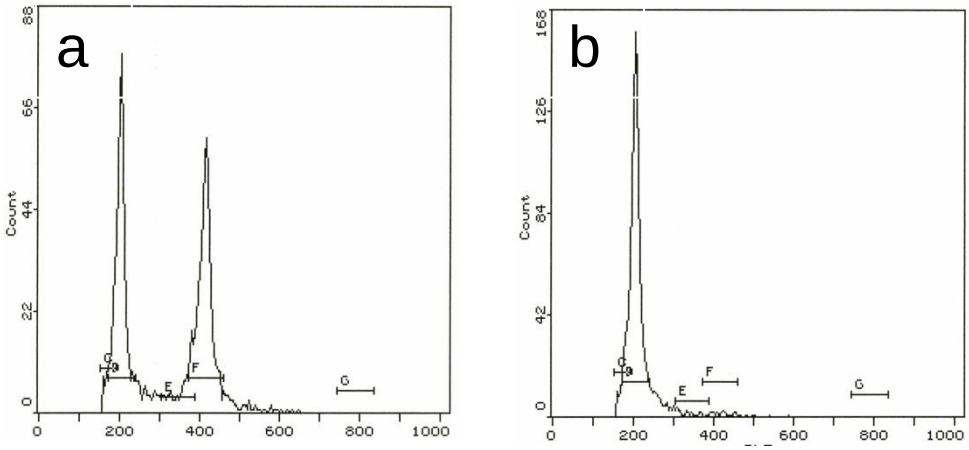

Figure 4.11 Histogram displaying the relative DNA content of leaf nuclei for a. SGR 1266 1.0% treatment and b. SGR 1266 diploid control bud sports.

The mixoploid condition of the leaves of the colchicine treated bud sports was consistent with the observation that some leaf areas contained smaller diploid-sized stomata, while others contained larger tetraploid-sized stomata.

Although mixoploid bud sports were detected, tetraploids were not detected from the flow cytometry analysis of the bud sports and therefore all clones had a zero tetraploid conversion.

CHAPTER 5
DISCUSSION

5.1 INTRODUCTION

In this study, to induce polyploidy, seeds were germinated in various colchicine concentrations for different times. During germination the fully established shoot apical meristem, from which the hypocotyl is derived, and root apical meristem, from which the root is derived, start to grow through mitotic division. The radicle of the developing embryo penetrates the surrounding structures of the embryo and imbibes water containing colchicine (Bewley, 1997; Finch-Savage and Leubner-Metzger, 2006). During this early development phase of an embryo a first round of cell division occurs during imbibition and a second round shortly after radicle elongation, which marks the start of seedling growth (Bewley, 1997). Exposure to colchicine during this period of rapid mitotic division most likely ensures maximum effectiveness of colchicine on cells from both meristematic regions. Polyploidy can thus result in both the shoot and root of the developing seedling.

Axillary buds also contain meristematic tissue. Because these meristems also contain numerous actively dividing cells, it is expected that treatment by colchicine would cause spindle inhibition and consequently produce polyploid cells. Depending on the number of polyploidy cells and which layers of the apical meristem are affected, the developing bud sport, could be completely polyploid or mixoploid (partially polyploid).

In this research project both seed and axillary buds were treated with different colchicine concentrations and exposures to induce polyploidy. The overall results of these inductions are presented in Table 5.1.

Table 5.1 Summary of the main findings of this investigation.

Species	Number of putative polyploids (%*)	Number of tetraploids	Number of mixoploids	Confirmed tetraploidy	Overall species survival
Seedlings					
E. urophylla	17 (12)	6	11	4.2%	52%
E. grandis	5 (4)	1	4	0.8%	63%
Bud sports					
E. grandis (2 clones)	6 (17)	0	3	0.0%	N/A
E. grandis hybrids (4 clones)	12 (17)	0	4	0.0%	

*Percentage putative polyploids identified during pre-screening calculated out of the total screened.

5.2 PHENOTYPIC EFFECTS OF POLYPLOIDY

In the treatment of seed the toxic nature of colchicine has shown to affect survival dramatically with increased concentrations and lengthy exposures (Thao *et al.*, 2003; Khosravi *et al.*, 2008; Lehrer *et al.*, 2008). However, treatments displaying lowest survival are also those treatments that have been most successful in inducing polyploidy (Khosravi *et al.*, 2008). When the overall seedling survival of the two species investigated was compared, it was found that survival in *E. urophylla* was appreciably less than *E. grandis* (Table 5.1). The treatments that were most successful in inducing polyploidy were also those that resulted in the lowest survival rates (Table 5.2). It could be concluded from these data that *E. urophylla* is more sensitive to the mutagenic effects of colchicine than *E. grandis*, exhibiting a lower survival rate, but in turn producing more polyploids (tetraploid and mixoploid) than *E. grandis* (Table 5.1). Thus, when attempts are made to

induce polyploidy, the best balance between survival and induction success should be sought.

A range of phenotypic expressions have been attributed to polyploidy. Other studies have shown that cells of polyploid seedlings tend to divide slower, and therefore have slower rates of root and shoot growth compared with the diploid counterpart (Lehrer *et al.*, 2008). This retardation effect becomes greater with increased concentrations and exposure times of colchicine (Upadhyaya and Nooden, 1977). Results from both *E. urophylla* and *E. grandis* tetraploid seedlings are consistent with this generalisation, displaying shorter and thicker roots and hypocotyls (Table 5.2), similar results were obtained with *Acacia mearnsii* seedlings (Moffet and Nixon, 1960). It was noted that the treatments that affected the root and shoot growth the most were also those that induced the most tetraploids or mixoploids in the seedlings exposed to those treatments (Table 5.2).

Table 5.2 Summary of the seedling induction responses to the various colchicine treatments.

Species and organs affected	Colchicine concentration and exposure affecting organs the most	Treatment inducing most polyploids*	Mean survival for each treatment**
E. urophylla roots	0.05%; 18 h 0.05%; 24 h	0.05%; 18 h 0.05%; 24 h	41% 30%
E. urophylla shoots	0.05%; 18 h 0.05%; 24 h		
E. grandis roots	0.05%; 18 h 0.03%; 24 h 0.05%; 24 h	0.03%; 24 h 0.05%; 24 h	45% 34%
E. grandis shoots	0.05%; 18 h 0.03%; 24 h 0.05%; 24 h		

*polyploids = mixoploids and tetraploids
**The mean survival for the treatment that induced the most polyploids

Variation in the responses between genotypes was detected within both species (Table 5.3). Different seedlots of a species appeared to be more sensitive to the mutagenic effect of colchicine, while others were more resistant (Table 5.3). It was also within the sensitive group that successes with polyploidy induction were obtained. The exception was U-140 that appeared to be resistant, but also produced two mixoploids. These results indicated that root and shoot growth responses to colchicine treatments for various genotypes may be indicators of polyploid tissue in both species, but could not distinguish between tetraploidy and mixoploidy.

Table 5.3 Summary of seedlot induction responses to the various colchicine treatments.

Species and organs affected	Seedlots most affected (sensitive)	Seedlots least affected (resistant)	Seedlots where polyploids* were produced	Seedlots where no polyploids* were produced
E. urophylla roots	U-123	U-135 U-140	U-123 U-124 U-125 U-138 U-139 U-140	U-116 U-135
E. urophylla shoots	U-125	U-116		
E. grandis roots	B4-54 B4-553 B4-873	B4-645 B4-689	B4-54 B4-415 B4-553 B4-873 B4-920	B4-645 B4-689
E. grandis shoots	B4-873	B4-689		

*polyploids = tetraploids and mixoploids

Several interesting leaf modifications were noted in the tetraploids and mixoploids. Generally tetraploid leaves were darker in colour than diploid leaves (Figure 4.8). As it has been shown in this investigation that polyploid cells possessed more

chloroplasts than diploid cells, it is probably justified to explain the increased depth in leaf colour by the increased number of chloroplasts and consequently an increase in chlorophyll concentration within the leaves manifesting as darker green leaves. This has also been observed in *A. mearnsii* (Mathura *et al.*, 2006) and *Colophospermum mopane* tetraploid plants (Rubuluza *et al.*, 2007).

A comparison of the leaf index, the ratio of leaf width to leaf length, of diploids, tetraploids and mixoploids revealed differences between the different ploids (Figure 4.8). Some tetraploid leaves were narrower and longer than the diploid leaves, thereby displaying a smaller leaf index; similar to what has been found in *C. mopane* tetraploids (Rubuluza *et al.*, 2007). This is, however, in contrast to what is generally expected with polyploids, which tend to have larger cells and thus larger organs (Mizukami, 2001). The mixoploids and other tetraploids, on the other hand, had a similar leaf index to that of their diploid counterparts.

Leaf margins of many of the mixoploids and tetraploids were generally asymmetrical and wavy (Figure 4.8). This was also observed in polyploids found in *Alocasia* (Thao *et al.*, 2003). In the mixoploids this could be explained by the different rates of cell division of the diploid and tetraploid leaf tissue (Otto, 2007). Diploid cells tend to divide quicker than polyploid cells, resulting in uncoordinated growth of the different leaf regions.

In mixoploid and tetraploid seedlings and mixoploid bud sports the expected enlargement of stomatal guard cells were observed (Mishra, 1997). In all instances, the stomatal guard cell sizes of polyploid seedlings and polyploidy tissue from the bud sports were significantly ($p<0.0001$) larger than those from their diploid counterparts (Table 4.2 and Table 4.8). In seedlings, the larger stomatal guard cells produced larger stomata with reduced frequency ($p<0.001$) (Table 4.6). An increase in stomatal guard cell size was also accompanied by a significant ($p<0.001$) increase in the number chloroplasts (Table 4.7). Similar relationships between the change of stomatal guard cell length, stomatal frequency and stomatal chloroplast frequency with an increase in ploidy level have also been observed in *A. mearnsii* (Beck *et al.*, 2003a; Beck *et al.*, 2003b; Beck *et al.*, 2003c).

A known tetraploid, an interspecific hybrid of *E. grandis* and *E. camaldulensis*, produced by Shell was included in this investigation to serve as a reference. The stomatal characteristics of the tetraploid (refered to as a reference tetraploid) and its corresponding diploid (refered to as a reference diploid) were used as a comparison to the induced material of the pure species. The induced tetraploid seedlings of *E. urophylla* and *E. grandis*, and the reference tetraploid, all exhibited similar stomatal characteristics; increased stomatal guard cell size (Table 4.2), decreased stomatal frequency (Table 4.6) as well as an increase in the stomatal guard cell chloroplast frequency (Table 4.7). When comparing the measurements of stomatal guard cell size (Table 4.2), frequency (Table 4.6) and stomatal chloroplast frequency (Table 4.7) between all tetraploids and between diploids, the measurements for the reference tetraploid and diploid were dramatically different to that of the *E. urophylla* and *E. grandis* tetraploid and diploid measurements.

The difference between stomatal guard cell size, frequency and stomatal chloroplast frequency of the reference material and the species could be attributed to the genomic constitution of the hybrid. Because of the mixed genomic constitution of the hybrid it is impossible to predict how these genomes, that are not naturally associated, will interact with one another (Nasrallah *et al.*, 2000). These data, therefore, demonstrate the importance of extensive testing of procedures before they are employed and the necessity to screen the different species and hybrids separately when screening for polyploidy.

These findings demonstrate that some phenotypic manifestations of polyploidy are not suitable to distinguish between the different ploids as the differences were inconsistent and undefined. Contributing factors to the indistinct phenotypic differences between the ploids include the fairly large genetic variation that existed amongst the seed used in the investigation and the expected variation in number of polyploid regions versus the diploid regions in the mixoploid leaves, blurring the differences between the ploids. However, stomatal guard cell size, stomatal frequency and number of stomatal chloroplasts are phenotypic expressions of polyploidy that are suitable for preliminary screening of induced *Eucalyptus* material, especially for seedling induction.

5.3 INDUCTION AND DETECTION OF POLYPLOIDY

Success of polyploidy induction using seed or axillary buds is directly attributable to the position of the cells mutated in the meristematic tissue and number of cells involved in the mutation. Mutations in the periphery of the meristematic tissue result in sectional and mericlinal mutations (Lineberger, 2008). From stomatal assessments these mutants will be identified as being mixoploid, possessing islands of tetraploid tissue amongst diploid tissue of leaves (Figure 5.1 a and b). The mixoploids produced in the bud sports in this research exhibited these islands and were therefore either sectional or mericlinal chimeras. These are highly unstable chimeras, in which the polyploid tissue is usually replaced by faster growing diploid tissue. In time the polyploid tissue disappears completely having being replaced by the diploid tissue (Szymkowiak and Sussex, 1996). This “replacement or displacement” phenomenon was also observed in chimeras in the induction of polyploidy in *Alocasia* (Thao *et al.*, 2003).

Mutations in the centre of the meristematic tissue result in periclinal mutants (Lineberger, 2008). From the stomatal assessment on the mixoploid seedlings the epidermis of leaves were fully tetraploid, unlike the bud sport leaves, and therefore were classified at periclinal chimeras (Figure 5.1 c). The probability that the resultant mutant is a complete polyploid depends on the number of cell layers involved in the centre of the shoot apical meristematic tissue in the mutation process (Lineberger, 2008). In the case of the induced tetraploids, the colchicine treatments allowed for maximum penetration of the inducer into the seed, therefore affecting multiple histogenic cell layers and therefore producing tetraploids (Figure 5.1 d).

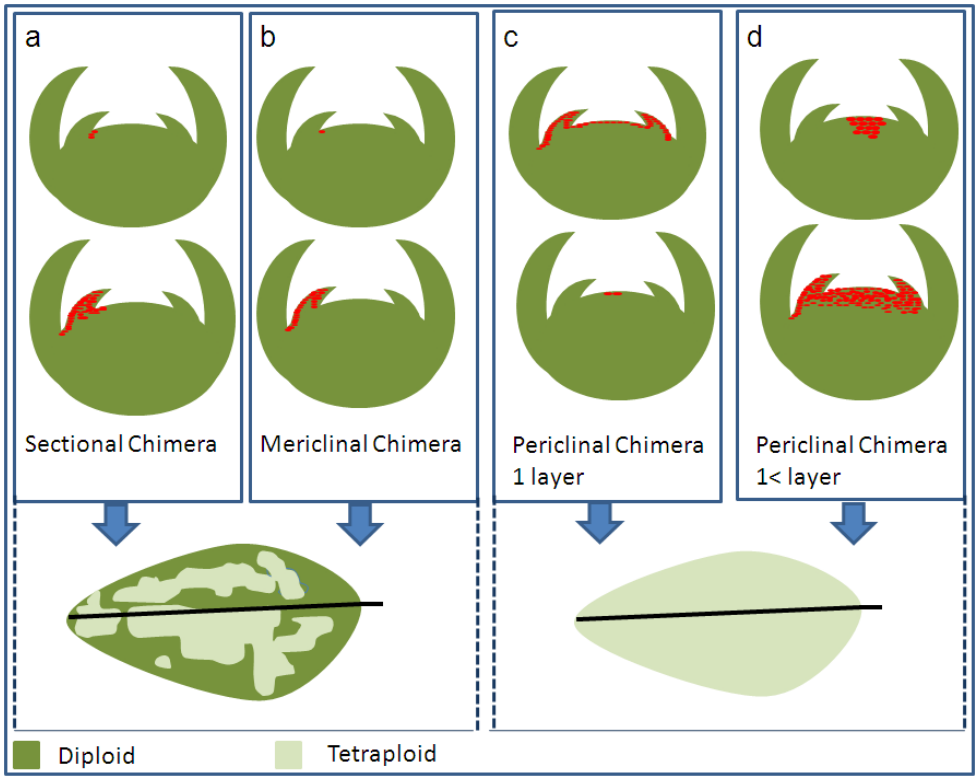

Figure 5.1 **Leaf phenotypic manifestations of various mutations in the apical meristematic tissue. a. Mutations in multiple cell layers of the peripheral cells produces a sectional chimera. b. Mutations in cells of a single peripheral cell layer produces a merclinal chimera and. c and d. Mutations in central cells that produce periclinal chimeras and tetraploids (adapted from Lineberger, 2008).**

It should be noted that periclinal mixoploid chimeras could have polyploid tissue derived from histogenic layer II. The cells in this histogenic layer are responsible for the development of the gametes (Dolen and Poetig, 1998); therefore these mixoploids can be of great value in crosses in a breeding programme. A periclinal mixoploid chimera with polyploidy tissue derived from histogenic layer II could be crossed with a diploid plant to form triploid individual plants and therefore demonstrates the need to determine what type of mutant a particular mixoploid is, because of the potential value of periclinal mixoploids in subsequent crosses.

5.3 CONCLUSIONS AND RECOMMENDATIONS

In this research project, tetraploidy was successfully induced in *E. Urophylla* and *E.grandis* seedlings. The highest number of tetraploids and mixoploids were induced by 0.05% colchicine for 18 h and 24 h exposure in *E. urophylla* and in *E. grandis* 0.05% and 0.03% colchicine for 24 h exposure. These treatments therefore represent the most optimal combinations of colchicine concentration and exposure time for polyploidy induction for this experiment. Although the seedlings from these treatments exhibited low survival rates it is recommended for future experiments that the most optimal concentration and exposure time be established to maximise polyploidy induction without compromising on seedling survival.

Although the success was limited in the induction of polyploidy in axillary buds, the results of this research reiterate the importance of extensive testing. Furthermore, because of the lower success rate of polyploidy induction in axillary buds compared with seed (Table 5.1), the use of seed is recommended in future induction experiments to maximise results. In the case where a particular genotype is being polyploidised, seed is not suitable and the use of other meristematic tissues should be investigated. To improve the rate of induction of polyploidy in non-seed tissue more research is required. Factors such as the dissipation of colchicine, exposure time and size of meristematic bud should be considered. Smaller buds have the potential for or a greater proportion of cells to be exposed to the mutagen and consequently a greater chance for a sport to be a complete mutant.

It could be concluded from these results that pre-screening using stomatal size, frequency and stomatal guard cell chloroplast frequency is accurate in assessing the induction of polyploidy in seedlings in *Eucalyptus*. These pre-screening methods are relatively fast and relatively cheap. However, because pre-screening is unable to distinguish between tetraploids and mixoploids, the pre-sreening should be followed-up with a DNA or chromosome quantification. In *Eucalyptus* with its small chromosomes, flow cytometry proved to be a suitable confirmation method.

The efficacy of polyploidy induction and verification requires a systematic approach. The following steps are recommended to maximise the success:

Steps	Process	Discussion/reasoning
Step 1	Undertake an extensive review of the literature.	Source information about potential mutagenic agents, concentration and range of mutagenic agents, exposure times and plant tissue needs.
Step 2	Select the most appropriate mutagenic agent, concentration and range of exposure times and suitable plant tissues.	Select information sourced from the species or related species. If no information exists about the species or related species a range of treatments should be tested.
Step 3	Undertake a pilot study.	A pilot study should be undertaken to narrow down the treatment range and method of application of mutagenic agent.
Step 4	Apply the optimised treatments identified in Step 3 to induce polyploidy.	Induction of polyploidy undertaken.
Step 5	Pre-screen induced material for potential polyploids.	Suitable pre-screening methods identified during the literature review should be employed to identify putative polyploids.
Step 6	Confirm polyploidy.	Confirm polyploidy using the most applicable methods; either through cytogenetic chromosome counting or quantification of DNA using flow cytometry.
Step 7	Allow polyploids to grow until strong and established.	Polyploids are often slow growing and need time to establish. During this time polyploidy detection techniques should be frequently perfomed on the confirmed polyploids to assess their stability.
Step 8	Assess polyploids for attributes for breeding purposes.	Polyploids must be assessed according to the original goal for their production.
Step 9	Multiply the polyploid vegetatively.	These valuable plants should be multiplied using methods suited to the particular species and then used in breeding programmes as required.

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APPENDIX

1. SOLUTION RECIPES

1.1 Seed sterilization solution

50% Jik® solution:

75ml of distilled water

75ml of Jik®

150 ml of 50% Jik® solution (10ml per seedlot)

1.2 Colchicine Solutions

1% stock solution:

1g colchicine was dissolved in 100ml distilled water.

0.01% colchicine solution*:*

10ml of 1% colchicine stock solution was added to 990ml of distilled water

0.03% colchicine solution*:*

30ml of 1% colchicine stock solution was added to 970ml of distilled water

0.05% colchicine solution*:*

50ml of 1% colchicine stock solution was added to 950ml of distilled water

1.3 Stomatal chloroplast staining solution

Iodine solution*:*

1g iodine

3g potassium iodide

Dissolved in 100ml of distilled water

1.4 Flow cytometry solutions

Otto I buffer (adapted from Otto, 1990)

4.2g citric acid monohydrate

1ml 1% (v/v) Tween 20

Adjust volume to 200ml to create a 0.1M solution

Otto II buffer (Otto, 1990)

28.65g Na_2HPO_4. $12H_20$

Adjust volume to 200ml to create 0.4M solution

1.5 Propidium Iodide (PI) solution

1mg ml^{-1} PI stock solution:

5mg PI powder was dissolved in 5ml distilled water

0.04 mg ml^{-1} PI staining solution: **(added to each sample)**

400µl of 1mg ml^{-1} stock solution was added to 10ml distilled water

2. EQUATIONS

2.1 Tetraploid conversion rate

$$\text{tetraploid conversion rate} = \frac{\text{number of identified tetraploids}}{\text{number of plants sampled}}$$

2.2 Polyploidy confirmation equation

sample ploidy =

$$\text{reference ploidy (external standard)} \times \frac{\text{mean position of the G1 sample peak}}{\text{mean position of the G1 reference peak}}$$