Plant Germplasm Conservation: Development of field Collection and Transport techniques for *Eucalyptus* species and *Trichilia Dregeana*

By

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PREFACE

The experimental work described in this dissertation was conducted in the School of Life and Environmental Sciences, University of Natal, Durban, from January 2000 to December 2001, under the supervision of Prof. Paula Watt, Prof. Patricia Berjak and Mrs Danile Macdonald.

These investigations represent original work by the author, while registered for the M. Tech: Biotechnology degree at Natal Technikon, and have not been submitted in any form for any diploma or degree to another Technikon or University. Where use was made of the work of others it has been duly acknowledged in the text.

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ABSTRACT

Lack of suitable techniques for field collection of the germplasm of different species, and spoilage of samples in transit, hinder efforts to collect, conserve, distribute and regenerate most plant germplasm *in vitro*. The aims of this investigation, therefore, were to address problems encountered in collection of field germplasm from species and hybrids of *Eucalyptus* (TAG5, TAG14, ZG14, GC550 and GU210) that are propagated vegetatively and *Trichilia dregeana*, which has recalcitrant seeds.

Simple *in vitro* culture-based protocols were developed to minimise contamination and maintain viability of plant material for sufficient time for it to be transported from the field to the tissue culture laboratory. From the two simulations of 48 h 'transportation' conditions for explants of *Eucalyptus* species investigated, those in bottles containing sterile vermiculite exhibited no contamination and greater than 50% bud break, regardless of whether or not field surface sterilization with alcohol had been done. In contrast, when explants were enclosed in cling wrap, contamination was high and bud break levels low.

For selection of the more suitable *Eucalyptus* explant, two types were investigated: nodal explants each with one half leaf (type 1) and stem segments with three nodes (type 2). As type 2 showed considerably better shoot yields (up to 1624 shoots per 100 explant), and were more practical to use with respect to space, such trinodal stem segments were deemed best for collection. Of the sterilization procedures investigated, treatment with 70% (v/v) alcohol prior to storage was found to be most suitable in almost all cases. For plant material with high endogenous microbial contamination, the bud break medium was supplemented with Benomyl and calcium hypochlorite, each at 0.5 and 1.0 $g.l^{-1}$. Alcohol-treated, stored explants cultured on bud break medium with 1.0 $g.l^{-1}$ calcium hypochlorite exhibited low levels of contamination and an increased final yield (up to a maximum of 930 shoots per 100 explants). Thus, this protocol was employed for field material of *E. grandis* clones TAG5, TAG14 and ZG14. For these clones, stored type 2 explants

subjected to alcohol treatment and calcium hypochlorite gave satisfactorily high yields (up to a maximum of 264 shoots per 100 explants were obtained).

Of the approaches investigated for *in vitro* collection of *T. dregeana* seeds, excision and direct placement of embryonic axes on sterile water agar medium in the field was most suitable. From several anti-microbial agents tested to reduce fungal contamination, a combination of an antibiotic with two fungicides in the water agar medium proved best. Surface-sterilized embryonic axes cultured on this medium in the field showed no contamination, and when transferred onto medium containing MS nutrients, 30 $g.l^{-1}$ sucrose and 10 $g.l^{-1}$ agar without anti-microbial supplements, 100% germination was obtained with only 15% of the specimens showing contamination.

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ABA	abscisic acid
ANOVA	one-way analysis of variance
BAP	benzylaminopurine
B5	Gamborg's (1968) nutrient formulation
°C	degrees Celsius
d	day(s)
FAP	furfurylaminopurine, kinetin
Fig	figure
g	gram
$g.l^{-1}$	gram per litre
$kg.cm^{-2}$	kilogram per square centimetre
$HgCl_2$	mecuric chloride
ha	hectares
h	hour(s)
IAA	indole-3-acetic acid
IBA	indole butyric acid
IRRI	International Rice Research Institute
L	litre
$\mu E.m^2.s^{-1}$	micro Einsteins per metre square per second
m	metre
min	minutes
ml	millilitre
$ml.l^{-1}$	millilitre per litre
$mg.l^{-1}$	milligram per litre
mm	millimetre
MS	Murashige and Skoog (1962) nutrient medium formulation
n	sample number
NAA	naphthalene acetic acid
%	percent/percentage
pH	hydrogen ion concentration
PVP	polyvinylpyrrolidone
s	second(s)
spp.	species (plural)
Tween 20	polyoxyethylene sorbitan monolaurate

v/v	volume per volume
w/v	weight per volume
ZEA	zeatin

1. GENERAL INTRODUCTION AND LITERATURE REVIEW

1. Plant tissue culture and *in vitro* conservation techniques

Plant tissue culture is the science of growing plant cells and tissues or organs isolated from the mother plant on artificial media. It includes techniques and methods appropriate to research in many botanical disciplines and offers several practical objectives (George, 1993). The conservation of plant genetic resources is a complex task encompassing various activities such as collecting, storage and evaluation of plant materials that have been collected. Various complementary approaches, including *in situ* and *ex situ* components are being developed to improve plant genetic resources conservation (Ashmore, 1997; McFerson, 1998).

The most commonly employed *ex situ* conservation method is the storage of seeds. However, as a number of species, particularly tropical or subtropical, have recalcitrant seeds or are predominantly vegetatively propagated, their conservation in seed form is impossible or has limited application (Berjak *et al.*, 1996). In such cases, species have been conventionally conserved as genebanks in the field. However, field genebanks are faced with serious problems including natural disasters, attacks by pests and pathogens and, in some cases, high labour costs. An alternative approach is the use of *in vitro* culture techniques, which involves the utilization of tissue culture methods to maintain (i.e. store) and produce plant material under sterile conditions (Berjak *et al.*, 1996; Ashmore, 1997).

The important factors of *in vitro* storage are the development of suitable techniques for (1) field collection, (2) *in vitro* plant regeneration protocols and (3) development of storage procedures for germplasm of different species.

Efforts to collect, conserve and distribute germplasm of plant species are often hindered by deterioration of samples in transit. Therefore, it is necessary to develop simple procedures for sterilisation in the field, which require only rudimentary equipment to be

transported to the field-site. *In vitro* storage is commonly applied to vegetatively propagated species and those with recalcitrant seeds, or in circumstances where transport would be unavailable or too costly (Yidana *et al.*, 1987; Ashmore, 1997). *In vitro* collection is the initiation of tissue cultures in the field for the purpose of initiating growth and for transport of material/samples back to the laboratory. Plant material that can be used for this purpose includes preformed meristems (e.g. shoot tips, embryos, leaf and stem pieces) (George, 1993; 1996; Ashmore, 1997). Field collection techniques range from performing surface sterilisation (using alcohol) and culture initiation in the open air to working within a sterile bag or box. For example, in the case of *Cocos nucifera*, zygotic embryos have been isolated and sterilised partially in the field and transported *in vitro* back to the laboratory (Assy-Bah *et al.*, 1987).

In order for *in vitro* systems to be effective, they require efficient and reproducible plant regeneration procedures. These may employ either organogenesis, which leads to shoot differentiation with subsequent adventitious root formation (Lakshmi and Shobha, 1985) or somatic embrogenesis, which is a process analogous to zygotic embryogenesis, resulting in the production of bipolar structures which closely resemble seedlings (George, 1993; 1996).

To conserve and store germplasm of importance, *in vitro* methodology is beneficial for a number of reasons. Plant germplasm conserved *in vitro* is not subjected to any natural disasters, and is maintained (as far as possible) in a microorganism-free, confined environment. Thus, *in vitro* conserved material is often superior to field-grown material in terms of germplasm health as plant material is maintained under controlled conditions (Ashmore, 1997). *In vitro* techniques can offer the possibility for germplasm collection and storage in order to conserve plant material in danger of extinction and that of economical importance.

1.1 *In vitro* cell and tissue culture

1.1.1 Culture types

Plant tissue culture involves growing plant cells, tissues or organs isolated from the mother plant on an artificial medium (e.g. George, 1993; 1996; Ashmore, 1997). Plant tissue culture is made possible by the ability of the plant cells to grow, divide and regenerate into an entire plant, a property called totipotency (e.g. George, 1993; Lowe *et al.*, 1996). The term is commonly used to define all kinds of *in vitro* plant cell, organ and tissue cultures. For each selected type of tissue culture, all *in vitro* stages are carried out under sterile conditions (George, 1996).

Callus, cell suspension, protoplast and anther/ovule cultures are recognised as types of cultures that exhibit unorganized growth. Callus cultures are described as masses of undifferentiated cells, which are formed by aseptically transferring a sterile explant onto a nutrient medium supplemented with plant growth hormones, usually an auxin (e.g. Duncan and Widholm, 1986; Collin and Dix, 1990; Allan, 1991). Cell suspension cultures are composed of a population of isolated plant cells and minute clumps of cells dispersed in an agitated liquid medium (e.g. Collin and Dix, 1990; Allan, 1991), while protoplast cultures consist of isolated cells, each without a cell wall (e.g. George, 1993; Scarpa *et al.*, 1993).

Organ culture involves the aseptic isolation of whole plant parts with a definite structure (e.g. meristem, shoot tip or shoot, node, embryo or isolated roots), which are placed in culture (Duncan and Widholm, 1986; Collin and Dix, 1990; George, 1993). Somatic cells in culture may undergo organogenesis to produce shoots and roots or may engage in somatic embryogenesis to produce embryos, which then germinate in a manner similar to natural zygotic embryos and produce a whole plant.

1.1.2 The explant selection, preparation and maintenance of aseptic cultures

Pieces of whole plants that initiate *in vitro* systems are called explants. The type of culture to be initiated is determined by the explant type and the purpose of the proposed culture. An effective explant should have cells able to undergo cell division and showing morphogenetic plasticity (e.g. Warren, 1991; George, 1993). Meristematic or rapidly growing tissues perfectly meet the requirement of being an effective explant, whereas cells of mature tissues are morphogenetically determined and hence less suitable (Durzan, 1984; Warren, 1991).

There are various types of plant parts that have been used as explants including meristems, shoot tips, root pieces and nodes (Ammirato, 1986; George, 1996; Ashmore, 1997). Meristem culture systems are obtained when extreme tips of shoots are cultured; the larger shoot apices give rise to formation of shoot cultures and node cultures are an adaptation of shoot cultures (George, 1996). Zygotic axes and cotyledons excised from seeds are referred to as embryo cultures. Roots may also be isolated from parent plants to give rise to root cultures which can be induced to grow *in vitro* and form adventitious roots (Ammirato, 1986; George, 1996).

As plants growing in the field are susceptible to diseases and endogenous contaminants, surface sterilisation of explants prior to culture is an absolute necessity. Surface sterilants such as sodium hypochlorite are usually efficient in eliminating superficial contaminants (Warren, 1991; George, 1993). Pre-treatment with fungicides, biocides and insecticides prior to explant selection for *in vitro* culture are also often used to eliminate contaminants in culture (le Roux and van Staden, 1991; Warren, 1991; Gamborg and Phillips, 1995; Camargo *et al.*, 2001). An alternative procedure to prevent surface contamination is to obtain explants from *in vitro* grown culture, even though such cultures are not guaranteed to be completely aseptic (Herman, 1996).

A culture grown *in vitro* is manipulated under sterile conditions to decrease risk of contaminating explants, and strict maintenance of sterile conditions while subculturing *in*

vitro must be exercised at all times because plant nutrient media support microbial growth. Precuations include performing aseptic culture techniques in a laminar flow cabinet and autoclaving media and utensils (De Fossard *et al.*, 1977; Gupta *et al.*, 1981; Dodds and Roberts, 1985; le Roux and van Staden, 1991; Warren, 1991; Gamborg and Phillips, 1995). It must be noted, however, that it is possible for the growth of endogenous microbial contaminants to be temporarily suppressed at initial stages of subculture (George, 1993; Herman, 1996; Ashmore, 1997).

Despite precautions, contamination by microorganisms is one of the major problems in plant tissue culture in scientific and commercial micropropagation laboratories (Leifert *et al.*, 1992, Leifert and Waites, 1992). Mites, thrips, fungi including yeasts, and bacteria have been reported to contaminate micropropagated plant cultures (Leifert *et al.*, 1989; Camargo *et al.*, 2001). According to Leifert *et al.* (1990) yeasts are the worst contaminants in plant tissue culture because of their insensitivity to low pH and tolerance of high sugar and salts concentrations, growth at low temperatures and tolerance to alcohol.

1.1.3 The culture environment

Many factors affect *in vitro* explant development and require consideration when such studies are undertaken. Furthermore, these factors are inter-related, resulting in an extremely complex situation (Ammirato, 1986; George, 1993).

The explant will grow *in vitro* when supplied with a specific medium formulation (George, 1996). A variety of media formulations for the culture of many different plant species have been devised and published [for example, White's medium (White, 1943), MS (Murashige and Skoog, 1962), Gamborg's B5 (Gamborg *et al.*, 1968)] (George *et al.*, 1988). They provide macro and micro nutrients that are needed for healthy and vigorous growth of the plant. To improve plant growth *in vitro*, media may also include trace amounts of certain organic substances notably vitamins, amino acids, coconut milk or

casein hydrolysate (Thorpe, 1980; Ammirato, 1986; Allan, 1991; George, 1993; Ashmore, 1997; Reid *et al.*, 1999; Stasolla and Yeung, 2001). Another factor, which greatly influences morphogenesis is the nature of gelling agent used, of which agar and Gelrite™ are the most commonly used agents (George, 1993; Beruto *et al.*, 1999).

The media are also supplemented with a carbon source because most *in vitro* culture systems are heterotrophic (Herman, 1996). Carbohydrates are commonly supplied as the carbon source usually in the form of sucrose, although the media may also be supplemented with monosaccharides such as glucose. Depending on species of interest and objectives of the investigation, the choice of carbon source may vary (Thorpe, 1980; Ammirato, 1986; Allan, 1991; Faure *et al.*, 1998).

Other important components are growth regulators that are used as supplements in the media. These play an important role in the induction and control of morphogenesis (Ammirato, 1986; Warren, 1991; George, 1996). Further, it is the ratio between plant growth regulators rather than the amount, which usually determines the route of development (Warren, 1991; George, 1996). The commonly used plant growth regulators in tissue culture are benzylaminopurine (BAP), indole acetic acid (IAA), furfurylaminopurine (FAP), naphthylacetic acid (NAA), zeatin (ZEA) and abscisic acid (ABA) and their properties and uses are reviewed extensively by Thorpe (1983), Ammirato (1986), Minocha (1987), Reynolds (1987) George (1996) and Bianco-Trinchant *et al.* (1999).

Browning of the medium at initial culture stages is a problem that is regularly encountered when using *in vitro* culture systems. This is due to the production of polyphenolics because of wounding, and can result in eventual death of the explant. This tends to be a serious problem for shoot cultures of hardwood species, such as *Eucalyptus* (MacRae and van Staden, 1990; Warrag *et al.*, 1990; Herman, 1995). Addition of adsorbents such as activated charcoal, polyvinylpyrrolidone (PVP), or antioxidants (e.g. ascorbic acid) to the nutrient medium has led to prevention of browning in the initial stages of many *in vitro* culture (Tuleckle, 1987; Warren, 1991; George, 1993). In

addition to chemical constituents of the culture environment, physical factors such as atmospheric gaseous consumption, light and temperature are also extremely important in development and morphogenesis of *in vitro* systems (Thorpe, 1980; Hughes, 1981; Ammirato, 1986, George, 1993). Obviously, plant development is dependent on respiration, which is also dependent on the availability of oxygen. An increase in oxygen supply was found to increase rate of respiration and development of potato explants (Forward, 1965; van der Plas and Wagner, 1986). Conversely, limiting oxygen supply can diminish plant growth rate (Kozai, 1991; de Goes, 1993; Shimazu and Kurata, 1999).

Light has a strong effect on plant morphogenesis in *in vitro* culture systems (Thorpe, 1980; Ammirato, 1986; Kozai, 1991). Some have different radiant energy requirements as compared with autotrophic plants, since the former do not photosynthesise (Thorpe, 1980); others, however, may be photosynthetic. Not only is the presence or absence of light important, but also the physical characteristics such as wavelength, intensity, quality and photoperiod are important to induce plant growth *in vitro* (Ammirato, 1986; Kozai, 1991; George, 1996). Although, the light required for photomorphogenesis and phototropism may be ignored (Thorpe, 1980; George, 1993), photosynthetic light is essential for promoting plant growth and producing better plant morphology (Kozai, 1991). Optimum light conditions have been found to vary from species to species (Ammirato, 1986).

Evaluations of temperature effects on tissue culture systems have not been thoroughly investigated. Generally, *in vitro* cultures are maintained at a constant temperature of around 25°C (Thorpe, 1980; Ammirato, 1986, George, 1993). It has been observed that the optimum temperature for growth varies from species to species and growth declines as temperatures are reduced from optimum (Grout, 1995*a*, George, 1996; Pliego-Alfaro *et al.*, 1996).

1.1.4 Applications of *in vitro* cultures

Biotechnology in its broadest sense is the management of biological systems for the benefit of humanity. It can offer an array of techniques including cell culture and micropropagation, *in vitro* genotypic selection, *in vitro* conservation and a wide number of technologies within the field of molecular genetics (Nel, 1985; George, 1993; 1996), for overcoming the conventional biological constraints of large size plants and the delayed sexual processes common to many woody species.

Micropropagation provides various advantages for the vegetative propagation of plants, including the short period required for the process to occur and less labour input than macropropagation methods. Additionally, micropropagation can be done throughout the year, plants *in vitro* are not subjected to any natural disasters, and desirable traits of the parent are retained. Moreover, if micropropagation is used in conjunction with conventional propagation methods, it can supplement clonal programmes in the rapid multiplication of selected genotypes (George, 1993, Ashmore, 1997, Watt *et al.*, 1997).

Somatic embryogenesis offers a number of improvements over micropropagation via axillary bud proliferation with respect to tree species. The most significant benefit of embryogenesis is the presence of both root and shoot meristem in the same unit. Other advantages include the potential to produce high yields of genetically uniform plantlets, synthetic seed production and a vehicle for genetic modification (Watt *et al.*, 1997)

In addition to the mass multiplication of elite genotypes, applications of culture systems with relevance to commercial forestry include cell suspensions as recipients for the insertion and integration of foreign genes, in the cryostorage of germplasm, and as systems for *in vitro* isolation of mutant lines and selection of stress- resistant phenotypes (van der Bulk, 1991). Furthermore, large scale automated production of somatic embryos from cell cultures in liquid media is a potential route for low cost plant multiplication (Becwar, *et al.*, 1988) and can overcome the problem of asynchronous embryo development encountered when solid medium is used (Watt *et al.*, 1991). Presently,

research applications are aimed at investigating the production of somatic embryos and calli, which are source material for genetic conservation and transformation studies (Watt *et al.*, 1997).

In vitro culture systems are basic to *in vitro* conservation, including both medium-term (minimal growth) and long-term (cryopreservation) approaches as discussed later.

1.2 *In vitro* conservation techniques

In vitro storage procedures fall into two categories, minimal growth (short-term storage) and cryopreservation (long-term storage). The term, minimal-growth, refers to growth inhibition or limitation (and other similar terms), which imply modification of the culture conditions, which result in slowed growth of the material *in vitro* (Withers, 1987). This type of storage is regarded as the most direct way of restricting growth and development of the *in vitro* material and is applied to differentiated plantlets and developing meristem cultures (Grout, 1995*a*). Minimal growth conditions have been used for the purposes of short to medium-term storage, but traditional germplasm banks can be substituted for only by the usage of long-term preservation practices such as cryopreservation (Withers and Engelmann, 1996). Cryostorage involves the reduction and subsequent arrest of all cellular divisions and metabolic processes of the plant material by storage at ultra-low temperatures, usually in liquid nitrogen (at $-196^{\circ}C$).

1.2.1 Collection techniques for conservation

Collection techniques include the possibility of selecting and gathering, and surface sterilising the plant material at the sampling site, temporarily storing it in sterile vessels, and then transporting it back to the laboratory (Assy-Bah *et al.*, 1987). Such an approach has been shown to be useful for collecting material of a number of species, including

cotton (*Gossypium* spp.) (Withers, 1985), cacao (*Theobroma cacao*) (Yidana *et al.*, 1987), and coconut (*Cocos nucifera*) (Assy-Bah *et al.*, 1987).

Methods of collection may vary according to the differences in the plant material. In the case of cacao and cotton germplasm, *in vitro* collecting techniques included disinfecting nodal cuttings in boiling water containing sterilisation tablets and fungicides, followed by inoculation onto semi-solid medium containing fungicides and antibiotics while the coconut embryos were isolated and rinsed once in sterile water and inoculated into culture on semi-solid medium in the field. In this way, the material was maintained for up to six months (Assy-Bah *et al.*, 1987; Yidana *et al.*, 1987).

In vitro collection techniques are not used in commercial plantation forestry and have not so far been tested for *Eucalyptus* germplasm (Watt *et al.*, 2000). The most common practice is to harvest branches from the parent plant in the field followed by the setting of cuttings in the nursery. Once these are established, they are then transferred to a greenhouse or to hedges where they serve as stock material for macropropagation and *in vitro* micropropagation (Watt *et al.*, 1997).

1.2.2 Disease indexing and microbial eradication

Even though surface sterilisation prior to *in vitro* culture eliminates some microbial organisms, frequently *in vitro* cultures or micropropagated plants have endogenous contaminants. To eliminate these, many different strategies are available, and may be used to remove or minimize surface and internal contaminants (Leifert and Waites, 1990; George, 1993; Barker and Terrance, 1997) before storage. Thermotherapy (exposure to 10-15°C above normal growing temperature), chemotherapy (treatment with anti-viral chemicals, fungicides and/or antibiotics) and meristem-tip culture (offering the possibility of virus elimination) are some of the specialised techniques employed to produce disease-free stocks (George, 1993; Horst, 1999). Nevertheless, further assessment must be performed to complement such techniques in checking for the presence of pathogens in

the tissue. Procedures for disease indexing may include techniques such as symptomatology, grafting/inoculation on indicator plants, ELISA and molecular techniques (Cassel *et al.*, 1980; Kartha, 1986; George, 1993; Barker and Terrance, 1997).

1.2.3 Propagation, characterisation, evaluation and monitoring

The propagation of plant germplasm is particularly important where large quantities of planting material are needed, or for the production of plants for use in breeding programmes or other activities (Ashmore, 1997).

In vitro propagation techniques allow for the rapid clonal multiplication of plants in a sterile, contained environment, independent of season and with very limited space requirement as compared to greenhouse and field propagation techniques (George, 1993; Ashmore, 1997). Where plant germplasm is conserved under medium term storage as *in vitro* cultures, propagation of plantlets can be carried out by subculture of material onto multiplication medium at times when plants are needed for either research purposes or distribution (George, 1993; Ashmore, 1997). However, culture conditions should limit the possibility of spontaneous genetic alterations (somaclonal variations) from arising. In some cases, somaclonal variation is reduced by limiting the number of cycles of propagation, decreasing levels of plant regulators, although cultured material will still need to be carefully monitored to remove any variant plants (Ashmore, 1997).

Micropropagation techniques are now available for a large number of plant species and, in many cases, the propagation of plants using tissue culture is on a commercial scale, as discussed previously (1.1.4). In managing a germplasm collection, plant material stored *in vitro* has to be well characterised. This is because the managers of the facilities have to be able to identify the germplasm, discern the relationships between accessions and genetic diversity in given gene pools, maintain the integrity of collections and locate and identify specific genotypes for use (Ashmore, 1997; McFerson, 1998).

To ensure and control the genetic stability of collections is important for germplasm conservation. Even though a high level of genetic stability is inherent in differentiated plant structures (e.g. buds, embryos), spontaneous genetic alterations are known to occur in undifferentiated material (e.g. callus) growing *in vitro* (Ahuja, 1987; Huang *et al.*, 1993). As a result, the preferred material to be maintained *in vitro* should be axillary buds, shoots, differentiated plantlets, embryos (zygotic and somatic) capable of continuing their development (Grout, 1995*a*).

In the past years, characterisation and monitoring tools used for germplasm collections have involved mainly morphological and agronomic traits, as the basis for separated accessions. This approach is based on the hereditary recurrence of certain genetically-controlled characteristics (McFerson, 1998). Techniques that have been used to characterise germplasm include, cytological techniques (e.g. karyotyping) and biochemical markers (e.g. isoenzymes) (Millar and Westfall, 1992). Recently, more sophisticated techniques have been introduced, such as molecular markers, which provide a new approach in locating genes of importance and gene complexes that code for more effective characterisation of conservation collections (McFerson, 1998).

1.2.4 Storage

Plant germplasm in *ex situ* conservation is maintained as either active or base collections (Hawkes, 1987). The active collection requires methods of storage that retain viability for short periods. These are normally maintained as field or greenhouse collections. The disadvantage of *ex situ* conservation collections is that they are extremely labour-intensive and therefore very costly. Also, substantial loss of material due to pests, diseases and environmental disasters may occur (George, 1993; Grout, 1995*a*; *b*; Ashmore, 1997).

For an *in vitro* storage system to be successful, it must have the ability to maintain the vigour and viability of material at the highest possible level and minimise the risk to genetic stability (Grout, 1995*a*).

Short-term storage methods have significant advantages over normal culture conditions as they avoid high cost and labour-intensive procedures that arise because of frequent sub-culturing (George, 1993). Species vary in the length of time for which their cultures can be stored. Periods varying from three (short-term) to 96 (medium-term) months have been cited in the literature (George, 1993). The types of explant used also contribute towards the storage period (Kartha, 1985; Withers, 1986; Grout, 1995*a*; *b*). Single shoots derived from actively dividing meristematic tissue have been found to be most suitable for storage due to the genetic stability of the tissue from which they are obtained (Henshaw, 1975; Kartha, 1985; Blakesley *et al.*, 1996). Additionally, unrooted shoot clusters, somatic embryos or rooted plantlets have also been found to be suitable for storage (George, 1993).

As mentioned previously, there are three *in vitro* approaches: a) storage of actively growing cultures, b) minimal growth (medium-term storage) and c) long-term storage (cryopreservation) (Withers, 1986; Bajaj, 1991; Krogstrup *et al.*, 1992; George, 1993; Hausman *et al.*, 1994; Grout, 1995*b*; Engelmann, 1997). The first method requires plant material to be maintained as actively growing cultures that need to be sub-cultured frequently, depending on growth rate of species. However, this method has an advantage because the plantlets may be rapidly multiplied by micropropagation. For minimal growth storage, tissue cultures are exposed to growth-limiting chemical or physical factors such as growth retardants, reduced temperatures and reduced pO_2 (Baucher *et al.*, 1989; Lizaragga *et al.*, 1989, Engelmann, 1990).

Cryopreservation is based on the reduction and subsequent cessation of metabolic functions in biological material while retaining viability, by applying extreme low temperatures (-80°C to -196°C) (Kartha, 1985; Berjak, 1996). Plant material can be preserved at such temperatures without alteration or modification for a theoretically

unlimited period. However, few biological materials can be frozen to subzero temperatures without adversely affecting cell viability (Kartha, 1985; Towill, 1985; Grout, 1991; Blakesley *et al.*, 1996; Engelmann, 1997), unless the effects of the complex manipulations are understood and problems overcome (Wesley-Smith, 2002). Cellular metabolic activities cease, when plant tissues are exposed to extreme low temperatures, as do most deleterious reactions, due to the absence of liquid water; consequently genetic aberrations are minimised. Cell suspensions (Withers, 1985), shoot and meristem cultures (Kartha, 1985) and somatic embryos (Withers, 1980) show potential for cryopreservation. Regeneration and functional potential of the stored germplasm must be maintained when it is returned to normal physiological environments, where growth and development *in vitro* must be minimised to allow extension of periods between subcultures. However, one has to make sure that labour input, materials and commitment of specialised growing facilities are economical and efficient (Grout, 1995*a*).

1.3 *Eucalyptus* germplasm conservation

1.3.1 The genus *Eucalyptus*: Botany and Economical Importance

The genus *Eucalyptus* is indigenous to Australia and also occurs naturally on the islands to the north of Australia including Timor, New Guinea and the Philippines (McComb and Bennet, 1986). It belong to the family Myrtaceae and are represented by about 800 species and hybrids (Zacharin, 1978). These vary in size from large broad-leafed trees to shrub-like mallees (i.e. *eucalyptus* scrubs flourishing in arid areas). According to Holliday (1973) the most distinguishing taxonomy feature is the presence of a fused calyx and/or corolla into an operculum. The operculum is hemispherical, the inflorescence is an umbel, the peduncle is distinctly flattened and the fruit capsule is about 5–11 mm in diameter. Eucalypts have smooth leaves and the adaxial surface is darker than the abaxial surface. The mature leaves are lanceolate, often falcate.

Forests are a highly significant component of South Africa's landscape and forestry is one of the country leading competitive industries. Plantation forestry (mostly of *Eucalyptus* and *Pinus*) is aggressively pursued in almost all provinces since natural forest species are slow growers and do not meet the South African demand for wood and pulp (van der Zel, 1989). *Eucalyptus* plantations for commercial production of wood in South Africa began about a century ago (Poyton, 1979; Darrow, 1984) and *Eucalyptus* species are now the most important commercial planted hardwood trees in South Africa (Graz and von Gadow, 1990). About 23 countries have areas of 30 000 ha or more planted with *Eucalyptus* and among those with more than 100 000 ha are South Africa, United States of America, Brazil, Australia, Argentina and Uruguay (van Wyk, 1990; Smith, 1996). A wide range of different *Eucalyptus* species is planted in South Africa and includes *E. nitens*, *E. macharthurii*, *E. smithii*, *E. grandis*, *E. saligna*, *E. dunni*, *E. paniculata*, *E. fastigata* and *E. diversicolor* as well as various hybrids. Naturally, *Eucalyptus grandis* occurs in a subtropical to warmer-temperate climate, which is humid throughout the year and in soils low in phosphorus and other nutrients (Poyton, 1979; Turnbull, 1991; Muller-Dombois, 1992; Denison and Kietzka, 1993; Smith, 1996). In 1990, alone, about 11,5% of the total area suitable for agriculture was utilized for plantation forestry in South Africa and 77% of the plantation sites was occupied by *Eucalyptus*, with *E. grandis* occupying about 29% of all commercial forestry land. In the period of 1991 to 1992, 19.8% of new afforestation in South Africa was achieved with *E. grandis* (Anon, 1993). Trees of this genus are economically important worldwide for production of oils, timber, pulp and paper. They are mainly grown for essential oil extracted from their leaves (Gupta and Mascarenhas, 1987); pulpwood used in the paper industry (van Wyk, 1990; le Roux and van Staden, 1991) and their timber which is used in mines (Lakshmi Sita, 1986; van Wyk, 1990; Watt *et al.*, 1991). Because of its rapid growth, adaptability and useful products, *Eucalyptus* is regarded as one of most productive plantation crops (Turnbull and Boland, 1984). However, the growth and distribution of *Eucalyptus* species is limited by climatic constraints such as temperature and water availability (Sommer and Wetzstein, 1984). In South Africa, the main factor limiting forestry expansion is the inadequate rainfall (Denison and Quaile, 1987).

In South Africa, *E. grandis* is mainly used for mining timber, and pulp and paper production. The pulp and mining industries account for 46% and 40% of the market consumption, respectively (van Wyk, 1990). In addition to pulp and paper production, South African companies (e.g. Mondi Forests and Sappi) produce eucalypts for woodchip and mining timber export (Smith, 1996). That author describes eucalypts as "super trees" because of their useful products that they provide to mankind. A wide range of different species is used for a variety of purposes such as production of industrial charcoal, domestic and industrial energy, sawn timber, essential oils, honey, tannin, shade and shelter, and leaves are used in animal fodder (Hills and Brown, 1978; McComb and Bennett, 1986; Turnbull, 1991; le Roux and van Staden, 1991).

Hybrids between *E. grandis* and other *Eucalyptus* species have been developed for many years in South Africa. These are becoming important for specific climatic regions of the country. Examples of hybrids include *E. grandis x E. europhylla*, *E. grandis x E. tereticornis*, and *E. grandis x E. camaldulensis*, for the subtropical regions, and for more temperate areas, *E. grandis x E. nitens* (Denison and Kietzka, 1993*a*). Development of hybrids of *E. grandis* has added considerable flexibility to the species. Heterosis (i.e. hybrid vigour) is usually the result and this is of great benefit to productivity of the forest industry. Further, growing sites can be extended to include drier and hotter areas, colder and more frost susceptible locations can be planted, greater disease resistance can be achieved and wood properties for specific end products can be improved (Denison and Quaile, 1987). The already existing high demands for *E. grandis* and hybrids are projected to increase markedly; therefore the need to optimise breeding techniques, wood quality and growth rate cannot be denied.

1.3.2 Propagation techniques

a) Conventional

Breeding and vegetative propagation of *Eucalyptus* species has attracted world-wide attention because of their desirable properties for fuel wood, pulp and paper. Forest demand is ever increasing and has resulted in the need for greater yields from plantations. A number of vegetative propagation techniques which have been improved over the years, have led to very significant results world wide, especially in Brazil (Ikemori *et al.*, 1994; Smith, 1996).

Vegatative propagation techniques can lead to successful clonal plantations of superior trees that produce better and greater yields than seedling-propagated plantantions (Zobel and Ikemori, 1983). This is the outcome of exclusive selection of better genomes and cross-multiplying them for planting. Sexual hybridization of genotypes with desirable traits can also be used as an approach to produce better individuals through controlled breeding (Palmberg-Lerche, 1993; Dewald and Mahalovich, 1997; Denison, 1999). Therefore, both vegetative propagation and controlled breeding contribute to tree improvement, and thus should be combined to form an interlinked system.

According to Burley (1987), sexual recombination is an extensive and a very costly procedure that requires the identification of superior/dominant genotypes and subsequent combination of difficult cross multiplication to produce progeny for the next generation. However, hybridization has been of great help in South African forestry in enhancing tree plantations allowing areas that are known to be traditionally off-site, now to be planted. As examples of subtropical eucalypts, the most common and very successful hybrid combinations are *Eucalyptus grandis* crossed with either *E. camaldulensis*, or *E. urophylla* (Denison and Kietkza, 1993; Bandyopadhyay *et al.*, 1999; Denison, 1999).

Vegetative propagation, which involves both traditional and recent breeding techniques, has made the work for plant breeders much more efficient, is easier and provides various

advantages (Namkoog, 1989; Miller, 1993). Ahuja (1993) described vegetative propagation as a tool for breeding, and a means of multiplying for the direct planting of high genetic quality material. Whether the starting material is clonal or not, use of vegetative propagation offers the ability to preserve and replicate exact genotypes without sexual recombination (Namkoog, 1986; 1989), giving greater control over genetic diversity, which is a critical tool for germplasm conservation. In addition, vegetative propagation is also valuable for the international distribution of conserved germplasm. Alternatively, for cases where seeds may not be physically available, or difficult to store (e.g. for species producing recalcitrant seeds), vegetative propagation can serve as a conservation method (Hamilton, 1994).

There is a need to combine both conventional and modern methods of propagation to meet the increasing demand of high quality wood efficiently and in shorter periods (Campinhos and Ikemori, 1997; Zobel, 1993). In conventional practice, leaving a few good quality individuals to re-seed a cleared area can propagate most forest trees. *Eucalyptus grandis* and many other *Eucalyptus* species or hybrids flower abundantly and their seeds can be stored satisfactorily but their propagation by seedlings is not ideal where rapid regeneration is required. This is because of difficulties related to long life cycles of species before maturation (Ahuja, 1993). Targeted traits may also be lost because seeds give rise to new progeny that are slightly different from the parent plants (McComb and Bennett, 1986).

One method of vegetative propagation is grafting. It makes propagation possible for many timber trees including *Eucalyptus* species (McComb and Bennett, 1986). However, late grafting (after early spring) can have negative effects on levels of survival (Gardner, 1998): For example, out of 600 grafts made for *E. dunni*, 56.5 % survived after six months compared with 9.5 % survival of 410 grafts made for *E. nitens* and 32.5 % of 320 made for *E. smithii* (McComb and Bennett, 1986). To consider this method for propagation purposes, careful selection must be taken into consideration to choose the kind of graft and the stock (Hartney, 1980). It is also important to use seedlings of the same species if not the same stock material, to avoid the possibility of incompatibility.

Thus grafting is not always a recommended procedure for propagation (McComb and Bennet, 1986).

Vegetative propagation via rooted cuttings has been the most successful method used commercially for a number of eucalypts (McComb and Bennett, 1986; Zobel, 1993). However, cold-tolerant *Eucalyptus* species do not propagate easily, and the method is not efficient in producing a sufficient number of plants for progeny testing, as a limited number of shoots can be produced from each individual and rooting is inadequate for many species (McComb and Bennett, 1986). In addition, no standard method exists for selecting cuttings for individual species, thus making the route highly inefficient for propagation.

b) Micropropagation

In vitro techniques such as micropropagation may be used in conjunction with macropropagation (George, 1993; Watt *et al.*, 1997) to overcome some of the difficulties discussed above. Micropropagation is a process that involves the multiplication and maintenance of selected genotypes *in vitro* under sterile conditions. This approach is widely used for agricultural crops and is also applied to forest species (le Roux and van Staden, 1991). Micropropagation results in very high production rates of selected tree genotypes, with resulting short-term silvicultural gains (le Roux and van Staden, 1991; Watt *et al.*, 2000).

Micropropagation is often used as a direct means to produce propagules in *Eucalyptus* in commercial forestry, to supply cuttings to clonal programmes, and for plantation establishment (Watt *et al.*, 1997). Furthermore, *in vitro* systems are essential for the regeneration and multiplication of new genotypes produced through recombinant DNA technology manipulations (Watt *et al.*, 2000). Based on published observations, it is suggested that micropropagation is a viable strategy for the mass production of plantlets for use in plantations and/or clonal hedges (Watt *et al.*, 1995). For *Eucalyptus* species

and hybrids, and those of other tree species, other methods for micropropagation have also been established, as discussed in section 1.2.3. However, micropropagation via axillary bud proliferation is the better-established method. It is used routinely in numerous research and commercial laboratories, including the Trahar Centre of Mondi Forests in KwaZulu-Natal (Watt *et al.*, 1995).

c) Germplasm storage

Utilization of *in vitro* techniques for germplasm storage is important for a number of reasons. Woody species (e.g. *Eucalyptus* spp. and *Pinus* spp.) for commercial forestry require large tracts of land to maintain conservation stands for breeding and clonal programmes (Watt *et al.*, 2000). Such extensive utilization of field space can now be avoided by using *in vitro* storage procedures as discussed in section (1.2).

Ex situ conservation is mainly used for agricultural and commercial forestry species. It involves the use of field plantations, seed stores and gene banks. *In vitro* storage techniques play an important role in the conservation of clones which are required as stocks for continued propagation *in vitro* or as parents in plant breeding programmes (Wilkens *et al.*, 1982; Krogstrup *et al.*, 1992; Grout, 1995*a*; *b*; Ashmore, 1997).

Ahuja (1993) describes synthetic seeds, which are encapsulated explants, as an additional dimension to storage of shoot cultures *in vitro* for the conservation of commercially valuable trees such as *Eucalyptus* species. They are small and easy to handle, and are considered useful in the exchange of sterile material among laboratories worldwide (Ashmore, 1997). Moreover, synthetic seeds offer the opportunity to store genetically heterozygous plants, or hybrids with unique gene combinations. Buds are useful since they are genetically stable and, when propagated, give rise to true-to-type plantlets, thus ensuring genotype conservation. As for shoot cultures, storage of encapsulated buds under growth-limiting condition reduces the numbers of subcultures and thus reduces contamination risks, cost maintenance and subsequent loss of material. However, such

storage has limitations such as problems that arise due to loss of vigour in plantlets as a result of a prolonged storage period (Withers and Alderson, 1986) and, because of the use of growth retardants associated with minimal growth, genetic stability cannot be ensured (Withers, 1988; Ashmore, 1997).

Pollen can also be used and have been widely recognized as part of a broader strategy for conservation of plant genetic material for forest and fruit species (Withers, 1991; Grout and Roberts, 1995). When seeds of *Eucalyptus* species are used for propagation purposes, approximately 5-7 years are required before flowering, and heterozygosity leads to variation between trees (Lakshmi Sita, 1986). The storage of pollen rather than seeds, can therefore reduce the time taken to cross two individuals with desired traits for that particular species.

1.4 Development of technique(s) for field-treatment of particularly obdurate seeds

1.4.1 Recalcitrant seeds and their characteristics

Roberts (1973) was the first to introduce the term, recalcitrant, in his categorisation of seeds. So far, seeds have been categorised as orthodox, recalcitrant or intermediate (Chin, 1996). Orthodox seeds are those that usually undergo maturation drying as the final stage of their development and are shed in the dehydrated state, or, if shed relatively wet, can thereafter be safely dried to moisture contents in equilibrium with low relative humidities (Berjak *et al.*, 1996).

Vertucci *et al.* (1991) concluded that in such a state, the tissue water in orthodox seeds is generally non-freezable and such dehydrated seeds may be safely stored at sub-zero temperatures for extended periods. In contrast, recalcitrant seeds are shed in a hydrated condition, and will tolerate little water loss either before or after they are separated from the parent plant. The degree of desiccation sensitivity of recalcitrant seeds varies and is dependent on developmental status and rate of dehydration (Pammenter *et al.*, 1998) but

all have the common property of not being amenable to long-term, air-dry storage and seemingly many are also chilling-sensitive (Chin and Roberts, 1980). Because of the difficulties which are encountered in handling such seeds, and for a better understanding of the phenomenon of recalcitrance, it was suggested that factors that influence seed behaviour in the context of natural habitat or extrinsic influences on the individual species, be considered (Berjak and Pammenter, 1994).

The inherent characteristics of the seed in its environment are important in understanding recalcitrance and its level, or the degree of longevity, when compared with the desiccation-tolerant and orthodox seeds. For example, orthodox seeds acquire desiccation tolerance via a variety of protective mechanisms including the accumulation of non-reducing sugars and dehydrins which are proteins that are induced by abscisic acid, and are suggested to have the ability to prevent, or induce tolerance of, free radical attack (Leprince *et al.*, 1993). Inadequacies in these and other developmental aspects might help to explain desiccation sensitivity of recalcitrant seeds. Other properties considered vital in acquisition of desiccation tolerance and maintenance of the desiccated state include: the physical characteristics of cells and intracellular constituents; insoluble reserve accumulation; intracellular de-differentiation; efficient operation of anti-oxidant systems; accumulation of putatively protective substances (in addition to sugars) including late embryonic abundant proteins (LEAs), as well as of amphipathic molecules; the presence and role of oleosins, and the presence and operation of repair systems during rehydration (Pammenter and Berjak, 1999). Fundamentally however, recalcitrant seeds are highly metabolic and lack the ability to shut down this active state (Berjak *et al.*, 1984; Pammenter and Berjak, 1999), which is considered a basic cause of their desiccation sensitivity (Pammenter and Berjak, 1999).

Ellis and co-workers (1990) defined a third category of seeds, which they called intermediate, in terms of their post-harvest behaviour. These are characterised by their ability to withstand dehydration to levels compatible with air-dry storage, but are relatively short-lived and may be chilling sensitive in this state [e.g. *Coffea spp.* (Ellis *et al.*, 1990); *Carica papaya* (Ellis *et al.*, 1991*a*) and *Elaeis guineensis* (Ellis *et al.*, 1991*b*)].

It appears though, that not all seeds can be placed into one of the above-mentioned categories, as the water level loss tolerated may differ markedly among species (Farrant *et al.*, 1988; Berjak *et al.*, 1990; Tompsett, 1992; Finch-Savage, 1996). Such observations have led to the suggestion that neither orthodoxy nor recalcitrance is an absolute phenomenon and because the intermediate category seems extremely loosely defined, there may be a continuum of seed behaviour grading from that characterised by extreme desiccation tolerance through a decreasing ability to withstand dehydration stress, to that of extreme sensitivity to even the slightest loss of water (Berjak and Pammenter, 1994). A further dimension to the difficulties of categorizing post-harvest seed behaviour is added by chilling-sensitivity (Berjak *et al.*, 1996). Thus, when working with recalcitrant seeds, special care needs to be taken in developing protocols for harvesting, packaging, and distribution and short to medium-term storage since such seeds are very sensitive and deteriorate readily.

1.5 Methods of Seed Storage

The post-harvest behaviour of seeds determines the potential for their storage under various conditions (Berjak *et al.*, 1996). Ever since Roberts (1973) first classified seeds into recalcitrant and orthodox groups, no method has been developed for the long-term storage of intact, recalcitrant seeds, although, over the past two decades, the subject of the storage of recalcitrant seeds has been reviewed and carefully studied. The report of King and Roberts (1980) on the achievements and possible strategies for storage of recalcitrant seeds, made an extensive study of recorded data for both temperate and tropical species. Chin (1996) compiled a report on recalcitrant seeds worldwide. Most studies conducted indicated successful measures for short-term storage of recalcitrant seeds, but in the preceding two and half decades, none of the world-wide studies resulted in successful long-term storage. Problems encountered include large seed size and very high and variable moisture content, and associated fungus growth during storage (Chin, 1996).

Since recalcitrant seeds are desiccation-sensitive, they can neither be dehydrated nor can they be frozen in the hydrated state. Presently such seeds can be stored intact, only at the hydration level that characterises them at shedding. However, this is strictly a short-term option, being a matter of days to weeks at worst, and months at best, depending on the species involved, as inherent deterioration coupled with the activity of microbes results in viability loss (Pammenter *et al.*,1994; Berjak, 1996). Storage at slightly reduced water contents ('sub-imbibed' storage), while initially thought promising (Chin and Roberts, 1980), appears to be harmful at least in the case of highly sensitive seeds of *Trichilia dregeana* (Drew *et al.*, 2000).

Nevertheless partial desiccation techniques and treatment with fungicides, have extended the period of storage to as much as a year for a number of species. Of the various methods that have been attempted for prolonging lifespan in storage of recalcitrant seeds or explants; only four main types have been found to be moderately successful, *viz* (i) moist (hydrated) storage, (ii) partial desiccation (although this is questionable), (iii) controlled atmosphere storage and (iv) cryopreservation (Chin, 1996).

(i) Moist Storage Method

This approach is usually termed 'wet or hydrated storage' (Berjak *et al.*, 1989; Farrant *et al.*, 1988; 1989), and has been practised for many years for seeds of a number of crops including rubber (Ang, 1978; Chin, 1996). In some cases seeds are stored in a saturated atmosphere, while damp charcoal, sawdust or a mixture of the two, are the most common storage media. Temperature ranges for storage are from 4°C to 20°C, depending on the chilling sensitivity of the species. Ultrastructural and biochemical investigations of seeds of a number of wet-stored recalcitrant species have shown that organisation and activity within the embryo cells increases during wet storage in line with the onset of germination, and sooner or later leads to cell division and extensive vacuolation, after which deterioration begins (Berjak *et al.*, 1989; Farrant *et al.*, 1988; 1989, Pammenter *et al.*, 1994). Thus, wet storage conditions favour germination, which results in storage of

developing seedlings, rather than ungerminated seeds. The more immediately and rapidly germination takes place after shedding, the sooner will cell division and vacuolation followed by the onset of deterioration be initiated, these events being inversely related to longevity of seeds in hydrated storage (Farrant *et al.*, 1989). In addition, microbial proliferation is another inevitable problem associated with wet storage because of the moist and generally warm storage conditions (Berjak *et al.*, 1990; Mycock and Berjak, 1990; Berjak, 1996; Chin, 1996; Calistru *et al.*, 2000). Furthermore, desiccation sensitivity increases once germination has proceeded to the stage of extensive vacuolation, as the minimum and lethal water content levels increase (Farrant *et al.*, 1986; Berjak *et al.*, 1989; 1992) and, because additional water is not available during storage, viability loss proceeds rapidly from this stage. Lifespan in storage can therefore vary (less than 2-3 weeks for some tropical species and 2-3 years for more chilling-tolerant temperate species stored at lower temperatures (King and Roberts, 1980a; Suska and Tylkowski, 1980; Pritchard and Prendergast, 1986; Farrant *et al.*, 1989; Fu *et al.*, 1990; Tompsett, 1992). Even though proven more efficient than other approaches, wet storage provides only for the short-to- medium term, and its success is unpredictable because of various problems encountered. For the present research purposes, however this method was used for short-term storage (24 – 72 hours) of recalcitrant seeds of *Trichilia dregeana* while transporting them to the laboratory.

(ii) Partial Desiccation Technique

This method involves partial air drying of recalcitrant seeds at 20°C to a certain level of moisture content, which is still relatively high compared with that of orthodox seeds. It has resulted in the longest period reported for storage of some recalcitrant species. Rubber seeds for example, have been processed, cleaned, treated with fungicides and subsequently surface-dried to a whole seed moisture content of just above 20% before storage. Cocoa seeds were processed by Hor (1984) by reducing their moisture content to 35-53% by drying them in an air-conditioned room (22°C and 55% RH). The seeds were dusted with a fungicide mixture, after being partially or surface-dried, and packed in

batches of 500 seeds in a thin (0.15mm) perforated polythene bag (200 mm x 300 mm). These were then put into bags and stored in a loosely closed box in an air-conditioned room. They retained a germination level of over 50% for up to 24 weeks, which was an improvement over the imbibed/moist method for this species and so far this has been the most successful and economical method demonstrated for storing rubber seeds (Chin, 1996). It needs to be appreciated, however, that while whole seed moisture contents were reduced, those of the embryonic axes (which constitute only a tiny fraction of the seed mass and volume) are generally maintained at considerably higher levels (Berjak *et al.*, 1996). However, other studies have cast doubt on the efficacy or universal applicability of this approach for storing recalcitrant seeds (Drew *et al.*, 2000). Success with this approach is more likely to be achieved consistently only for non-orthodox seeds that are not truly recalcitrant (e.g. intermediate seeds), or, it recalcitrant, only it the embryonic axes are resistant to water toss, and/or if partial desiccation effectively curtails fungal proliferation.

(iii) Controlled Atmosphere Storage

Maintenance of seeds using this method involves storage in various gases or in sealed containers; however, this approach has shown little success in prolonging life span (Chin, 1996). By just waxing the whole pod, Pyke *et al.* (1934) illustrated that seeds of cocoa could be stored, but this is very different from seed storage *per se*, as the seeds are possibly preserved and/or kept from germinative metabolism by the surrounding fruit tissues. Even though maintenance under controlled atmospheres has been tried, results indicate only a very short-term solution, making this method of limited practical application in the storage of recalcitrant seeds (Chin, 1996).

(iv) Cryopreservation (Long-term storage method)

Storage of recalcitrant seeds at sub-ambient temperatures (with other conditions as described in i, ii and iii) has been found to be inappropriate for many species, at least for long-term storage. However, cryopreservation, which involves sample storage at very low temperatures (usually in liquid nitrogen at -196°C), is attracting an increasing interest world-wide for a long-term storage of seeds (if they are very small) or of seed components. These techniques share a similar approach to those found to be successful in the preservation of other biological samples *viz.* plant meristems, mammalian embryos, semen and ova which have been discussed as models for excised recalcitrant embryos (Chin and Roberts, 1980).

Cryostorage offers the option for long-term conservation of recalcitrant species. It obviously prevents germination and also microbial growth and obviates physiological deterioration in the tissues, since all cell metabolic processes are inactived. Theoretically, cryopreservation can facilitate storage for thousands of years (Ashwood-Smith and Friedman, 1979, Dumet *et al.*, 1997; Engelmann, 1997). However, lethal ice formation is likely to occur if care is not taken to adjust seed moisture contents to suitably low levels when below-zero temperatures, and certainly those lower than −18°C, are used. For successful storage of seeds at very low temperatures, they must be partially dried prior to freezing so that they will not suffer freezing injury before being cryopreserved in liquid nitrogen (Berjak, 1996; Wesley-Smith, 2002). Although cryostorage is promising, whole seeds can only very rarely be used. This is because of their necessarily high water contents, and usually large size, which makes successful dehydration and freezing impossible. Thus, as an alternative, cryopreservation of excised embryonic axes was investigated for long-term storage of the germplasm of species producing recalcitrant seeds. Excised embryonic axes are found to be more tolerant to dehydration than are whole seeds, principally because they are very small, allowing the water to be withdrawn very rapidly. This does not allow the time for lethal damage to occur (Pammenter *et al.*, 1998), as is the case during the necessarily slow dehydration of larger structures.

Withers (1980) and Bajaj (1985) suggested that, for vegetatively propagated plants and species with recalcitrant seeds, germplasm could possibly be conserved through cryopreservation of the excised embryos or their segments. Sun (1958) and Withers (1980) have showed that dehydrated materials exhibit remarkable resistance to cryogenic damage. However, generally in such studies, the degree of desiccation applied could itself be lethal (Wesley-Smith *et al.*, 1992; Wesley-Smith, 2002). Accounts of cryopreservation of embryonic axes for both temperate and tropical recalcitrant species have been reported, but on examination of the published data, Berjak *et al.* (1999) are sceptical of the true meaning of the success claimed.

1.6 The need for collection of recalcitrant seeds, and *in vitro* germplasm preservation

Many of the plantation crops, fruit and timber species in the tropics, produce recalcitrant seeds. Over 70% of timber species in rainforests are suspectedly recalcitrant (Ashton, 1982; Chin, 1996). The requirement for conservation of germplasm of such species is well recognized, as there is no method for long term storage of recalcitrant seeds compared with orthodox seeds for which seed banks exist world-wide (Chin, 1996).

According to that author, another reason to stress the necessity for conservation of recalcitrant species, is that they may become extinct. Conservation of tropical rainforest species, which are relatively inaccessible, presents many problems as most are very large, perennial trees, and additionally, produce seeds that are recalcitrant and thus short-lived. Seeds of such species are comparatively large, soft and often chilling-sensitive, so cannot be stored in cold rooms. *In situ* methods such as conservation in national parks and forests, expose the species to many environmental hazards (Chin, 1996), and *ex situ* plantations occupy large tracts of valuable land.

In comparison, the species producing orthodox seeds are mainly annual plants, exemplified by most of the food crops, where propagules of thousands of varieties of a

particular species can be stored in a seed bank; for example, at the germplasm centre of the International Rice Research Institute (IRRI) in Los Baños, in the Philippines.

According to Chin (1996) commercial timber species, exemplified by members of the family Dipterocarpaceae, produce recalcitrant seeds. This family is particularly prominent in Malaysia and the East generally. Requirements in Malaysia for reforestation programmes, amount to 30 million seeds per annum, and to procure and store such great numbers of recalcitrant seeds even on a short term basis, is very difficult (Chin, 1996). Already in 1973, Karma stressed the problem of poor and insufficient seed storage in the developing countries of the tropics, and additionally there is no system of standard certification of stored recalcitrant seeds as is available for orthodox seeds (Perera, 1973; Chin,1996).

1.7 Germplasm conservation of *Trichilia dregeana*: an example of a tropical species producing recalcitrant seeds

The Meliaceae is a large family of trees and shrubs with species found in Africa, Asia and America. Two tree species of *Trichilia viz T. dregeana* and *T. emetica* are found in South Africa. Both of these species are beautiful, shady trees with useful timber (Pooley, 1993) and both produce recalcitrant seeds (Kioko *et al.*, 1998).

Trichilia dregeana Sond. is a magnificent tall tree reaching heights of 30 m or more. It is found in high rainfall, evergreen coastal forests in eastern areas of KwaZulu-Natal and the Eastern Cape provinces (Pooley, 1993). The tree is not endemic to these areas and occurs in much of east Africa. The fruits are roundish capsules and ripen from March to mid- year. The entire capsule is then abscised, when generally, the tenuous connection with the fruit wall is broken, resulting in a localized scattering of seeds. Up to six large seeds, each of which is almost completely covered by a scarlet aril, occur per capsule (Pooley, 1993).

Generally, bees, butterflies and moths feed on the flowers of *T. dregeana*. The fruits are eaten by birds and seeds may be used to produce oil. Other parts of the trees such as leaves, roots and bark are used for medicinal purposes. In the early 1800s the timber was used to repair ships entering the port of Durban. Furthermore, several species of butterfly breed on this tree including the white barred *Charaxes*. The tree grows quickly from fresh seeds (Pooley, 1993). The problem though is that the seeds of *T. dregeana* are recalcitrant (Choinsky, 1990; Kioko *et al.*, 1998) and preservation of the germplasm is the subject of intensive research at the University of Natal in Durban. However, these efforts are frequently hampered by significant infection and poor seed quality or production. The present research aims to establish better collection techniques in the field, and storage of seeds while in transit, to decrease levels of deterioration of the material and contamination.

1.8 Aims of the investigations

This investigation was aimed at addressing problems that are encountered in collection of field material for *in vitro* storage and micropropagation purposes. In particular, attention was focused on germplasm from *Eucalyptus* species and hybrids (which can be propagated vegetatively) and *Trichilia dregeana* seeds. In both cases, these species usually occur in habitats (plantations or natural, respectively) far from the storage and propagation laboratories and, consequently, germplasm may be lost in transit.

For *Eucalyptus* species and hybrids, the objectives were to establish: (a) the most suitable explant type for collection; (b) appropriate sterilization procedure in the field prior to collection; and (c) transportation conditions using both fungicide-treated (greenhouse-grown) and non-fungicide-treated (grown outside the greenhouse) plant material. The established protocol was then tested on field material.

For recalcitrant seeds of *T. dregeana*, the objectives were to develop: (a) pre-sterilization protocols for seeds/embryonic axes in the field; and (b) suitable transportation conditions from the field to the laboratory.

2. MATERIALS AND METHODS

2.1 Studies on *Eucalyptus* species and hybrids

2.1.1 Plant material and maintenance of parent plants

Cutting-derived stock plants of *Eucalyptus grandis* (TAG5, TAG14 and ZG14), *E. grandis x citriodora* (GC550) and *E. grandis x urophylla* (GU210) were used. Stock plants of TAG5 were obtained from Mountain Home Laboratory, Mondi Forests, Hilton and the others were obtained from the Mondi Forests nursery at Kwa-Mbonambi (KwaZulu-Natal, South Africa).

a) Fungicide-treated stock plants

Parent plants were maintained in the greenhouse at the University of Natal, Durban. They were watered daily. Plants were also sprayed with fungicides and fertilizers on a weekly basis. The fungicide treatments included foliar spraying with mixtures of 2% (w/v) manganese ethylenebis (dithiocarbonate) polymeric complex with zinc salt (Mancozeb; Efetkto, South Africa) and 0.1% (v/v) tetrachloroisopthalonitrile (Chlorothalonil; Shell S.A., Johannesburg; South Africa) and application to the soil of 0.1% (w/v) *N*-propyl-*N*-[2,4,6trichlorophenoxyethyl]imidazole-1-carboxyamide (Prochloraz; Hoechst Scherig AgrEvo, Johannesburg, South Africa) and 0.125% (w/v) 1H-1,2,4-triazol-3-ylamine (Triazole; Bayer, Johannesburg, South Africa).

Fertilizer applications were 0.25% (v/v) trace element solution (Trelmix; Hubers, South Africa) applied as a foliar spray or alternatively, 0.1% (w/v) (1N-2P-1K) (Mondi Orange; Harvest Chemicals, South Africa) was applied to the soil. The parent plants were cut back every three to four weeks to stimulate coppice growth.

b) Non-fungicide-treated stock plants

These stock plants were grown in potting bags outside the greenhouse, fertilized and watered, as described above, but not treated with fungicides.

c) Field-grown plant material

Stock plant material of *Eucalyptus grandis* (TAG5, TAG14 and ZG14) were maintained at Mondi Forests nursery, KwaMbonambi (KwaZulu-Natal, South Africa). Plants of TAG5 and ZG14 were grown in open fields and relied on rainfall for their water. They were supplied with fertilizer only once, around the stumps, after coppice collection. TAG14 was grown in hedges, watered and supplied with fertilizers through computer-controlled dripper lines daily.

2.1.2 Optimisation of explant selection, pre-storage treatment

2.1.2.1 Collection and pre-treatment of explants

Sprouted branches of all the *Eucalyptus* material (approximately 100 mm in length) with preformed apical and axillary buds were harvested and cut into two types of explants, *viz.* (1) one nodal explant with one half leaf (20 – 35 mm in length) (explant type 1) and (2) stem segments (35 - 50 mm in length) with three nodes and no leaves (explant type 2) (Fig. 2.1). Half of these were then either treated with 70% (v/v) alcohol or not, after which they were placed in moistened vermiculite or enclosed with cling wrap as described below (2.1.2.2) and transferred to the laboratory. The remaining explants were either alcohol-treated or not (as mentioned above) but were not placed in vermiculite or cling-wrapped and were taken straight to the laboratory where they were immediately surface sterilized and placed into culture.

2.1.2.2 Simulated transport storage conditions

Two storage conditions for samples in transit were simulated. After pre-treatment of explants they were prepared as follows: (a) five explants of type 1 (one nodal explant with one half leaf) or type 2 (stem piece with 3 nodes) were placed upright with the cut end inserted into 3 – 4 g moist sterile vermiculite in 250 ml culture bottles (Fig. 2.2). These were then closed and maintained for 48 hours in the dark at 25 ± 2°C. (b) Six sprouted branches (approximately 150 mm) were sprayed lightly with sterile distilled water, then wrapped loosely using cling wrap (Fig. 2.3) and placed for 48 hours in the dark at 25 ± 2°C.

2.1.2.3 Culture conditions

Once explants were received in the tissue culture laboratory, they were surface sterilized in 0.02% (w/v) $HgCl_2$ containing two drops of Tween-20 for 10 min and then rinsed three times in sterile distilled water. Explants were then placed in 1% (w/v) calcium hypochlorite for 2 min, rinsed with sterile distilled water three times, and trimmed to fit culture bottles. In these bottles, explants were placed into bud induction medium containing MS nutrients (Murashige and Skoog, 1962), 0.1 $mg.l^{-1}$ biotin, 0.1 $mg.l^{-1}$ calcium pantothenate, 0.04 $mg.l^{-1}$ 1-naphthylacetic acid (NAA), 0.11 $mg.l^{-1}$ 6-benzylaminopurine (BAP), 0.05 $mg.l^{-1}$ 6-furfurylaminopurine (FAP), 20 $g.l^{-1}$ sucrose and 4.0 $g.l^{-1}$ Gelrite™). In certain experiments, the medium was supplemented with calcium hypochlorite and Benomyl® at (1.0 and 0.5 $g.l^{-1}$ respectively). The cultures were checked for microbial contamination every 48 h and non-contaminated explants transferred into fresh, sterile medium. At the end of the bud break period (ten days) contamination and bud break were quantified on a percentage basis.

Explants were then transferred into multiplication medium containing MS nutrients, 0.1 $mg.l^{-1}$ calcium pantothenate, 0.2 $mg.l^{-1}$ 6-benzylaminopurine (BAP), 0.1 $mg.l^{-1}$ biotin, 0.01 $mg.l^{-1}$ 1-naphthylacetic acid (NAA), sucrose and 4.0 $g.l^{-1}$ Gelrite) for four to five

weeks, after which contamination, multiplication and average number of shoots per explant were assessed and counted at the end of the multiplication stage.

The pH of all media was adjusted to 5.6 – 5.8 prior to autoclaving for 30 min in a TOMY autoclave (120 – 125°C, 1.0 – 1.5 $kg.cm^{-2}$). All cultures were maintained at a 16 h light (200 $\mu E/m^{-2}s^{-1}$)/8 h dark photoperiod at 23 to 25°C.

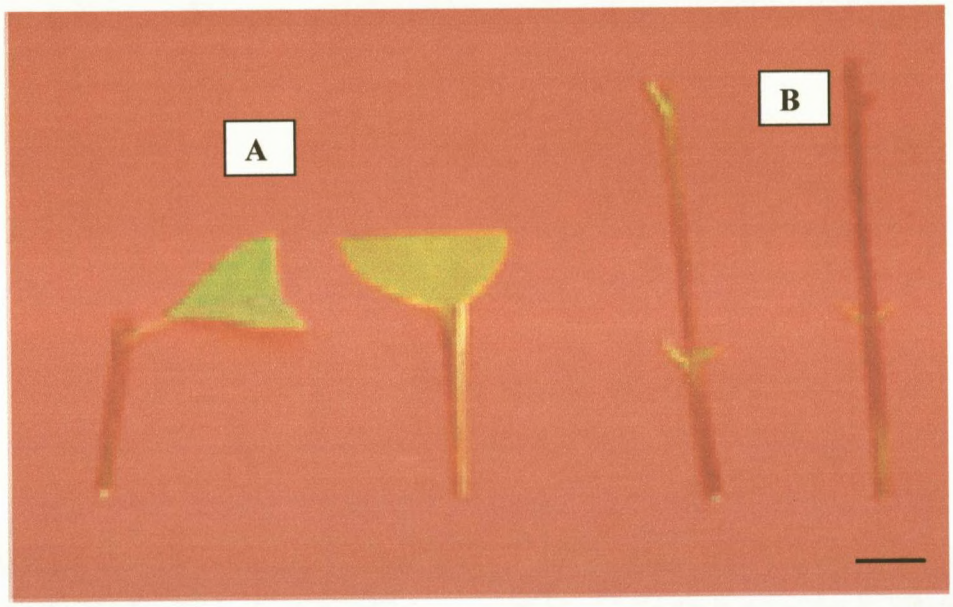

FIGURE 2.1: Types of shoot explants collected *in vitro* and used in storage (A) Explant type (1) one nodal explant with one half leaf (20 – 35 mm in length) and (B) Explant type (2) stem piece with three nodes and no leaves (35 - 50 mm in length) [bar = 3.2 mm].

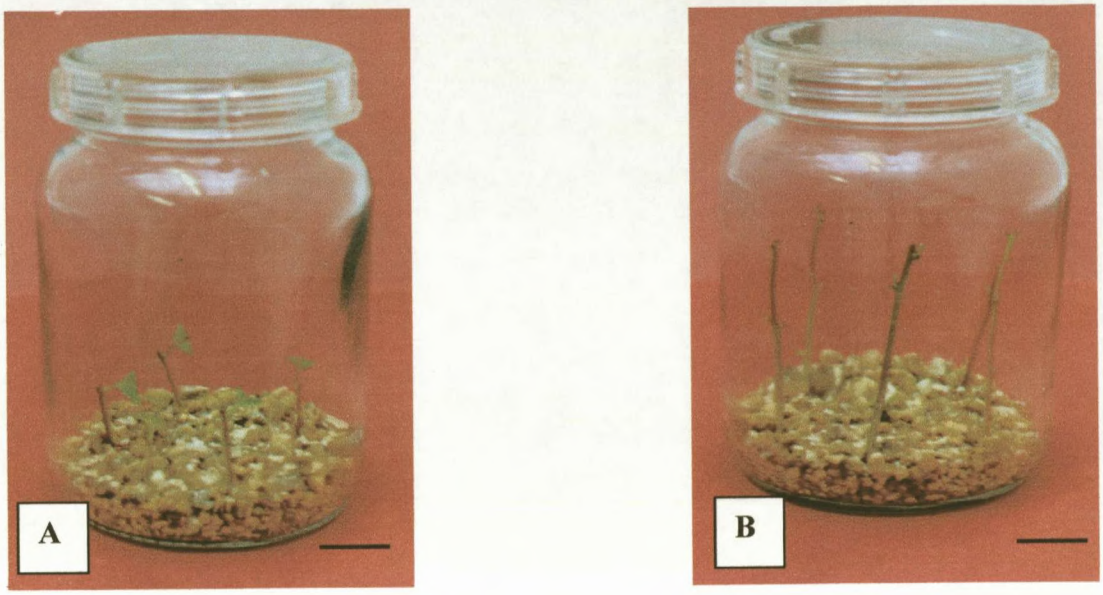

FIGURE 2.2: ***In vitro*** **storage of explant type 1 (A) [bar = 3.3 mm] and explant type 2 (B) [bar = 3.4 mm] in sterile moistened vermiculite (maintained for 48 h in the dark at 25 ± 2°C).**

FIGURE 2.3: ***In vitro*** **storage of eucalypts branches in cling wrap (maintained for 48 h in the dark at 25 ± 2°C) [bar = 2.1 mm].**

2.2 Studies on *Trichilia dregeana*

2.2.1 Preparation for short-term storage at the collecting site

a) Batch 1

Seeds were collected directly from the trees growing on grassy verges of side streets of Durban in the mid-morning. All subsequent work was done under shade and using as aseptic conditions as possible in the field, to minimise contamination. Using a scalpel, the waxy aril of the seed was removed manually (which is vital, as many fungal propagules are harboured in this waxy covering). Seeds were accumulated in a beaker covered with a moistened paper towel (to prevent dehydration) until required numbers were obtained. Seeds were then rinsed briefly with sterile distilled water, and transferred into sterile temporary storage salt solutions, either potassium chloride, potassium sorbate, or sodium benzoate (Saarchem, South Africa) at 16 $g.l^{-1}$ in impermeable plastic containers. The seeds (n = 60) were then transported in each of these solutions from the collecting site to the laboratory. Seeds were sampled for *in vitro* culture after 24, 48 and 72 h in the transport solutions to test for viability and contamination levels.

b) Batch 2

After collection and aril removal as in (a) above, the seeds were surface-sterilised in 1% (v/v) sodium hypochlorite for 20 min. Sodium hypochorite was discarded, then seeds transferred into temporary storage salts without any rinsing and were transported to the laboratory and sampled as in (a) above.

c) Batch 3

Seeds were collected and cleaned as in (a) above. They were then surface-sterilized in 1% (v/v) sodium hypochlorite for 20 min, rinsed three times in sterile distilled water and then soaked for 2 min in a fungicide cocktail (i.e. a mixture of 0.2 $ml.l^{-1}$ Impact; (Zeneca

Agrochemicals, South Africa) and 2.5 $ml.l^{-1}$ Previcur N (AgrEvo, South Africa), then rinsed briefly in sterile distilled water, after which the seeds were transferred into temporary storage salt solutions, and transported to the *in vitro* laboratory and sampled as in (a) above.

2.2.2 Germination and contamination assessment

After storage in temporary salt solutions as above, half of the seeds (n = 30) were briefly re-sterilized with 1% (v/v) sodium hypochlorite, rinsed and set to germinate at a depth of approximately 10 mm in moistened vermiculite. The germination trays were maintained at 25 ± 2°C under 12 h light (intensity unassessed)/dark conditions, and watered regularly. Germination was scored when shoots emerged. For the other half of the seeds, the embryonic axes were excised. The axes were then surface sterilized for 10 minutes using 1% (v/v) sodium hypochlorite under aseptic conditions in the laminar hood. These were then cultured for 30 days in growth medium containing MS nutrients, 30 $g.l^{-1}$ sucrose and 10 $g.l^{-1}$ agar for contamination assessment, which was assessed visually.

All cultures were incubated at 16 h light (200 $\mu E/m^{-2}s^{-1}$)/8 h dark photoperiod at 23 to 25°C. The pH of all media was adjusted to 5.6 – 5.8 prior to autoclaving.

2.3 Development of a direct technique for introduction of embryonic axes into culture in the field

Seeds were collected in the same manner as previously described (2.2.1, above), and the waxy aril removed. Embryonic axes were excised and accumulated on moist filter paper in a closed Petri dish until required numbers were reached (n = 50 per treatment). All work was done under shade while camp gas burners were on during the entire procedure to eliminate/decrease contamination from the open field environment. Embryonic axes were surface sterilized using 1% (v/v) sodium hypochlorite for ten minutes, then rinsed

three times using sterile distilled water. These were then transferred into pre-prepared tubes of sterile semi-solid water agar, supplemented with a variety of anti-microbial agents. These were either fungicides, antibiotics or a mixture of the two *viz* (a) 0.2 $ml.l^{-1}$ Impact; (b) 2.5 $ml.l^{-1}$ Previcur N; (c) 0.64 $g.l^{-1}$ Kanamycin; (d) a mixture of Impact, Previcur N and Kanamycin (at above-mentioned concentrations); (e) 1.0 $g.l^{-1}$ Benomyl. After axes were introduced, culture-tube lids were closed and sealed using Parafilm. The axes were then transported to the laboratory in this state and cultured *in vitro* for a period of 10 d. Contamination levels were recorded and non-contaminated axes were transferred to growth medium containing MS nutrients, 30 $g.l^{-1}$ sucrose and 10 $g.l^{-1}$ agar (2.2.2), to assess viability by onwards growth of axes to the seedling stage, *in vitro*.

2.4 Data analyses and photography

Data were analyzed using a number of techniques. *In vitro* collecting experiments for *Eucalyptus* species and hybrids were repeated three times with 20 samples per collection of each species or hybrid. Where appropriate, data were analysed statistically using a one-way analysis of variance (ANOVA) and differences were contrasted using Scheffe's test and Duncan's multiple range test (at 95% confidence level). For studies on *Trichilia dregeana*, ANOVA and descriptive statistics in Microsoft Excel 97 were used. Photographs of explants for both *Eucalyptus* and *Trichilia* species that were regenerated *in vitro* were recorded with a Nikon FM2 camera, using AGFA films.

Table 2.3: Information regarding the antifungal/bactericidal agents used in this investigation (2.3).

Trade name	Active ingredient	Fungicide/ bactericide group	Example of target species	Manufacture/ Distributor
Previcur N	propamocarb-hydrochloride	carbamate	*Aphanomyces*, *Bremia*, *Plasmopara*, *Pseudoperonospora*, *Pythium*	AgrEvo, South Africa
Impact	triazole, benzamidazole	benzimidazole, (systemic and contact fungicide)	*Rhynchosporium*, *Puccinia*, *Erysiphe*, *Septora*	Zeneca Agrochemicals, South Africa
Benomyl WP	benomyl	benzimidazole	*Botrytis*, *Fusarium*, *Rhizoctoria*, *Verticillium*, *Sclerotinia*	Sanachem, South Africa
Kanamycin monosulphate	(kanamycin A)	antibacterial antibiotic	wide range of bacteria	Sigma-Aldrich Chemicals Co., South Africa

3. RESULTS

3.1 Development of a protocol for collection and storage: preliminary investigations on *Eucalyptus* material grown at the University of Natal (Durban)

For purposes of this study, preliminary investigations were performed using a variety of *Eucalyptus* species, clones and hybrids, some of which were known to have high levels of endogenous microbial contaminants. For practical reasons (i.e. distance from the field to the tissue culture laboratory), the initial studies were undertaken at the University of Natal (Durban) on parent plants grown either inside the greenhouse and treated routinely with fungicides (primarily to test if the proposed sterilisation methods had a deleterious or lethal effect on plant material) or outside the greenhouse and not treated with fungicides (mimicking the field conditions). The protocols developed were then tested on explants collected in the field. Material was monitored at the end of two culture stages. For stage 1, explants were cultured for a period of ten days to promote bud break, after which both this development and contamination were quantified on a percentage basis. The non-contaminated explants were transferred to stage 2 of culture, i.e. placed on multiplication medium for four to five weeks, when contamination and average number of shoots per explant were assessed.

The first investigation in this study involved two simulations of 48 h of transport conditions. Explants were either 'transported' in moistened sterile vermiculite within a closed tissue culture bottle (Figure 2.2) or enclosed in cling wrap (Figure 2.3). The effects of these conditions were compared in terms of contamination levels of explants and bud break percentage obtained after 10 days in culture. Studies then focused on determining the most suitable explant-type for collection, as well as the best sterilisation procedure in the field. The two explant types investigated were as shown in Figure 2.1. The surface sterilisation procedures used at the field were a+: (sprayed with 70% (v/v) alcohol); a-: (not sprayed with alcohol); s-: (cultured immediately, not stored) or s+: (stored) for 48 hours in sterile moistened vermiculite in the dark at room temperature.

3.1.1 Studies with *Eucalyptus grandis* plants (ZG14)

a) Comparison of storage methods

Initially, a comparison was done between two storage methods, i.e. use of vermiculite (Figure 2.2) or cling wrap (Figure 2.3), for samples in a simulated transportation condition. The results obtained showed that for both explant types 1 and 2, storage in sterile vermiculite within bottles was significantly better than in cling wrap (Table 3.1). In contrast to explants in cling wrap, where contamination levels were high and bud break low, those in bottles exhibited no contamination and had greater than 50% bud break, regardless of whether or not field surface sterilisation with alcohol had been done. Consequently, for all subsequent experiments, bottles containing sterile moistened vermiculite were used.

Table 3.1: Comparison of storage methods for clone ZG14 grown in the greenhouse and treated with fungicides. Pre-treatments: a+ = sprayed with 70% (v/v) alcohol, a - = not sprayed and s+ = stored for 48 h in sterile moisten vermiculite in the dark at room temperature. Explant types: 1 = nodal explant with one half leaf and 2 = stem piece with three nodes. Mean separation within columns ($p \leq 0.05$). n = 20. $^{a-c}$; data sharing same character are not significantly different.

Storage method	Explant type	Treatment	% contamination	% bud break
Bottle with vermiculite	1	a- s+	0	63^{c}
		a+ s+	0	51^{b}
	2	a- s+	0	67^{c}
		a+ s+	0	55^{b}
Cling wrap	1	a- s+	80	16^{a}
		a+ s+	50	20^{a}
	2	a- s+	75	18^{a}
		a+ s+	57	34^{ab}

(b) Greenhouse-grown plant material

Fungicide-treated (greenhouse-grown) plant material was used to establish whether the proposed sterilisation methods had detrimental effects on the explants or inhibited bud break.

The results for *E. grandis* (ZG14) indicated that both explant-types exhibited low contamination levels at bud break and multiplication stages, irrespective of treatments used, with exception of type 1 subjected to treatment a- s-, which had high levels of contamination (up to100%) at both culture stages (Table 3.2). Percentage bud break for all treatments was similar, except for explants of type 1 and 2 subjected to treatment a- s+, which resulted in a significantly higher bud break (63 and 67%, respectively). Shoot multiplication of type 1 stored explants treated with alcohol was relatively high (13.7 shoots per explant). Further, of the two explant types, type 2 yielded a significantly higher number of shoots (26.8 shoots per explant) with treatment a- s+, compared with explants of all other treatments.

As the value of a protocol to the industry is dependent on final yield and as explants are lost throughout the various culture stages, the final yield of multiplied shoots per 100 explants per treatment was calculated (Table 3.2). The highest final yield of 1662 shoots for stored explants was obtained with type 2, treatment a- s+. For material from the fungicide-treated plants, storage in vermiculite had no significant detrimental effect on explants or on subsequent multiplication. In fact, multiplication yield was increased for explant type 2, treatment a-. Storage had no effect on final yield (e.g. type 1 and 2, treated with alcohol) or significantly increased final yield (e.g. type 1 and 2, not treated with alcohol). In conclusion, final yields were similar for both explant-types subjected to all treatments with the exception of type 2 subjected to treatment a- s+. As these gave the greatest yield, and because these explants have three nodes (hence more material in each collection bottle), they are more practical to use with respect to space, whereas explant type 1 has one node for collection and storage.

Table 3.2: Effect of sterilisation and storage treatments on percentage contamination, percentage bud break, multiplication and final yield per 100 explants of two types of explants of fungicide-treated ZG14. Pre-treatments: a + = sprayed with 70% (v/v) alcohol, a - = not sprayed, s+ = stored for 48 h in sterile moistened vermiculite in the dark at room temperature and s - = not stored. Explant types: 1 = nodal explant with one half leaf and 2 = stem piece with three nodes. Mean separation within columns ($p \leq 0.05$). n = 20. $^{a-c}$; data sharing same character are not significantly different.

Explant type	Treatment	STAGE 1: BUD BREAK		STAGE 2: MULTIPLICATION		
		% contamination	% bud break	% contamination	No of shoots/explant	Final yield/ 100 explants
1	a-s-	93	50^{b}	100	0^{a}	0^{a}
	a-s+	0	63^{c}	0	10.5^{a}	662^{bc}
	a+s-	0	47^{ab}	33	18.2^{b}	573^{bc}
	a+s+	0	50^{b}	0	13.7^{a}	685^{bc}
2	a-s-	0	31^{ab}	27	19.3^{b}	437^{b}
	a-s+	0	67^{c}	6.7	26.8^{c}	1662^{c}
	a+s-	0	18^{a}	20	11.4^{a}	164^{ab}
	a+s+	6.7	55^{b}	0	7.14^{a}	365^{b}

(c) Materials grown outside the greenhouse

Having established that neither surface sterilisation nor storage in vermiculite for 48 h had significant phytotoxic effects on greenhouse-grown material, subsequent investigations were done on material grown outside the greenhouse. These non-fungicide-treated stock plants cultivated outside the greenhouse were used to mimic field material.

Results from non-fungicide-treated *E. grandis* (ZG14) (Table 3.3) indicated that both explant-types exhibited high levels of contamination (up to 100%), regardless of treatments used. Bud break was relatively low, the highest (53% of explants) being with

type 1 subjected to treatment a- s+. However, bud break yields obtained were considered to be meaningless since most of the explants were lost due to contamination, resulting in no explants being available for transfer to stage 2 in almost all cases.

Therefore, a modified approach was investigated to reduce contamination levels with this clone, involving supplementing the bud break medium with anti-microbial agents [i.e. Benomyl as used by Watt *et al.* (1996) or calcium hypochlorite as used by Yanagawa (2000) each at 0.5 and 1.0 $g.l^{-1}$]. These studies were done with type 2 explants only, for the reasons explained above (Tables 3.1 and 3.2). Anti-microbial agents, added to bud break medium at either concentration did not appear to have detrimental effects on stored or non-stored explants (Table 3.4).

Results presented in Table 3.4 indicated that usage of anti-microbial agents, at 1.0 $g.l^{-1}$ for non-stored explants resulted in relatively low contamination levels at stage 1 (6.7 and 20%, respectively), with an increase in stage 2 (22 and 44%). In contrast, stored explants exhibited high levels of contamination at both stages (up to 100%, stage 2) with the exception of explants cultured on bud break medium with calcium hypochlorite. At both concentrations, 0% contamination occured in stage 1, but contamination was evident in stage 2 (66 and 53%; 0.5 and 1.0 $g.l^{-1}$, respectively). Nevertheless, explants that were stored and cultured on medium with calcium hypochlorite, at both 0.5 and 1.0 $g.l^{-1}$, had a significantly high bud break of 67%. Storage did not appear to stimulate final yields (shoot yield per 100 explants) significantly, with the exception of stored explants cultured on bud break medium supplemented with calcium hypochlorite at 1.0 $g.l^{-1}$ (930 shoots). This was the result of a significantly higher shoot multiplication (30 shoots per explant) of stored explants cultured on medium with calcium hypochlorite at 1.0 $g.l^{-1}$, treatment a+ s+. Final yields from stored and non-stored explants for both calcium hypochlorite treatments were significantly higher than when Benomyl at either concentration was used (in particular, for stored explants, Benomyl at 0.5 $g.l^{-1}$ did not reduce contamination levels and no explants survived).

From the results obtained (Table 3.4) it was concluded that for successful storage, alcohol treatment and bud break medium supplemented with calcium hypochlorite were the best treatments with respect to multiplication and final yield.

Table 3.3: Effect of sterilisation and storage treatments on percentage contamination, percentage bud break, multiplication and final yield per 100 explants of two types of explants of non-fungicide treated ZG14. Pre-treatments: a+ = sprayed with 70% (v/v) alcohol, a - = not sprayed, s+ = stored for 48 h in sterile moistened vermiculite in the dark at room temperature and s - = not stored. Explant types: 1 = nodal explant with one half leaf and 2 = stem piece with three nodes. Mean separation within columns ($p \leq 0.05$). n = 20 $^{a-c}$; data sharing same character are not significantly different. * lost through contamination.

Explant type	Treatment	STAGE 1: BUD BREAK		STAGE 2: MULTIPLICATION		
		% contamination	% bud break	% contamination	No. of shoots/explant	Final yield/ 100 explant
1	a-s-	80	6.7[a]	100	*	*
	a-s+	73	53[b]	100	*	*
	a+s-	87	13[ab]	50	1.5	1.5
	a+s+	93	6.7[a]	100	*	*
2	a-s-	67	6.7[a]	100	*	*
	a-s+	100	0[a]	*	*	*
	a+s-	100	0[a]	*	*	*
	a+s+	87	0[a]	*	*	*

Table 3.4: Effect of anti-microbial agents, sterilisation and storage on percentage contamination, percentage bud break, shoot multiplication and final yield of shoots per 100 explants of explant type 2 from non-fungicide treated ZG14 plants. Explant type 2 = stem piece with three nodes). Pre-treatments: a+ = sprayed with 70% alcohol (v/v), a- = not sprayed, s+ = stored for 48 h in sterile moistened vermiculite in the dark at room temperature and s- = not stored. Mean separation within columns ($p \leq 0.05$). n = 20. $^{a-c}$; data sharing same character are not significantly different.

		STAGE 1: BUD BREAK			STAGE 2: MULTIPLICATION		
Treatment	Compounds	Conc. ($g.l^{-1}$)	% Contamination	% Bud break	% Contamination	No. of shoots/ explant	Final yield/ 100 explants
a+ s-	Benomyl	0.5	20	31^{ab}	44	11^{a}	154^{a}
		1.0	6.7	62^{bc}	21	15^{ab}	687^{b}
	calcium	0.5	13	44^{b}	38	16^{ab}	377^{ab}
	hypochlorite	1.0	20	33^{ab}	22	24^{b}	480^{ab}
a+ s+	Benomyl	0.5	73	20^{a}	100	-	-
		1.0	33	44^{b}	33	13^{a}	455^{ab}
	calcium	0.5	0	67^{c}	66	21^{b}	483^{ab}
	hypochlorite	1.0	0	67^{c}	53	30^{c}	930^{c}

3.1.2 Studies with TAG14, GU210 and GC550

Having established that *Eucalyptus grandis* (ZG14) explants could survive 48 h in storage, the investigations that followed on other clones were done only with non-fungicide-treated plant material grown outside the greenhouse to mimic conditions of field material. Plant materials used were a pure *E. grandis* clone (TAG14) and a clone of *E. grandis x urophylla* (GU210) and a clone of *E. grandis x citriodora* (GC550).

Both explant types of TAG14 exhibited relatively low levels of microbial contamination (maximum 27%) at stage 1 (Table 3.5). A significantly higher bud break was obtained with stored type 1 explants treated with alcohol (100% of explants) compared with all

other treatments. The number of shoots per explant was similar for both types with all treatments except for type 1, treatments a- s- and a+ s-. The highest final yield for stored explants was obtained with type 1 (375 shoots) and was significantly higher when compared with other yields, except for type 1, treatment a- s+ and type 2, treatment a- s-, where yields were not significantly different. For type 1 explants, storage seemed to stimulate final yield (shoot yield per 100 explants). For this clone, explant type 1, treatment a+ s+ appeared best with respect to bud break and final yield obtained.

Table 3.5: Effect of sterilisation and storage treatments on percentage contamination, percentage bud break, multiplication and final yield per 100 explants of two types of explants of non-fungicide treated plants (TAG14). Pre-treatments: a + = sprayed with 70% (v/v) alcohol, a - = not sprayed, s+ = stored for 48 h in sterile moistened vermiculite in the dark at room temperature and s - = not stored. Explant types: 1 = nodal explant with one half leaf and 2 = stem piece with three nodes. Mean separation within columns ($p \leq 0.05$). n = 20. $^{a-c}$; data sharing same character are not significantly different.

		STAGE 1: BUD BREAK		STAGE 2: MULTIPLICATION		
Explant type	Treatment	% contamination	% bud break	% contamination	No. of shoots/explant	Final yield/ 100 explants
1	a-s-	0	93^{b}	0	2.3^{a}	214^{ab}
	a-s+	6.7	87^{ab}	21	4.5^{b}	288^{bc}
	a+s-	20	80^{ab}	67	1.8^{a}	38^{a}
	a+s+	0	100^{c}	13.3	4.31^{b}	375^{c}
2	a-s-	6.7	84^{ab}	29	5.3^{b}	294^{bc}
	a-s+	13	84^{ab}	54	7.3^{b}	245^{b}
	a+s-	6.7	78^{ab}	36	4.8^{b}	226^{b}
	a+s+	27	58^{a}	45	3.5^{ab}	81^{ab}

With clone GU210 (Table 3.6), stored explants of both type 1 and 2 had low contamination levels at stage 1 (maximum 27%), although an increase was observed at stage 2 for most treatments. Bud break obtained with stored explants of type 1 treated with alcohol (60% of explants) was significantly higher than levels obtained with type 2 explants, regardless of treatments used. There were no significant differences among multiplication levels obtained with either explant type, except type 1 subjected to treatments a- s+ and a+ s+, which resulted in a significantly lower multiplication yields and type 2, treatment a- s+ where zero multiplication occurred. The highest significant final yields were obtained with non-stored explants of type 1 not treated with alcohol (120 shoots) and for stored type 2 explants treated with alcohol (135 shoots). There were high levels of contamination for explant type 2 at stage 2 (Table 3.6), and bud break percentage was low when compared to TAG14 (Table 3.5). It was then hypothesized that explants of this hybrid needed a longer period in culture for increased bud break.

Subsequent experiments were done using type 2, recording bud break at 10, 20 and 30 days. Results obtained indicated that storage and longer culture period significantly increased final yield from 85 to 870 shoots (s-) and from 135 to 1921 shoots (s+) (Tables 3.6 and 3.7). It was thus concluded that this clone did need longer culture period for maximum final yields.

Table 3.6: Effect of sterilisation and storage treatments on percentage contamination, percentage bud break, multiplication and final yield per 100 explants of two types of explants of non-fungicide treated GU210. Pre-treatments: a + = sprayed with 70% (v/v) alcohol, a - = not sprayed, s+ = stored for 48 h in sterile moistened vermiculite in the dark at room temperature and s - = not stored. Explant types: 1 = nodal explant with one half leaf and 2 = stem piece with three nodes. Mean separation within columns ($p \leq 0.05$). n = 20. $^{a-c}$; data sharing same character are not significantly different .

		STAGE 1: BUD BREAK		STAGE 2: MULTIPLICATION		
Explant type	Treatment	% contamination	% bud break	% contamination	No. of shoots/explant	Final yield/ 100 explants
1	a-s-	47	53^{bc}	25	5.7^{ab}	120^{c}
	a-s+	27	54^{bc}	50	1.8^{a}	36^{ab}
	a+s-	53	33^{ab}	57	5^{ab}	35^{ab}
	a+s+	27	60^{c}	45	3.8^{a}	92^{b}
2	a-s-	6.7	18^{a}	60	12.8^{b}	87^{b}
	a-s+	13	33.8^{b}	100	0^{a}	0^{a}
	a+s-	6.7	27^{ab}	78	15.5^{b}	85^{b}
	a+s+	27	44^{b}	20	5.25^{ab}	135^{c}

Table 3.7: Effect of sterilisation and storage treatments on percentage contamination, percentage bud break, multiplication, final yield per 100 explants and prolonged culture period (10, 20 and 30 days) of explant type 2 of non-fungicide treated plants (GU210). Explant type 2 = stem piece with nodes. Pre-treatments: a + = sprayed with 70% (v/v) alcohol, a - = not sprayed, s+ = stored for 48 h in sterile moistened vermiculite in the dark at room temperature and s - = not stored. Mean separation within columns ($p \leq 0.05$). n = 20. $^{a-c}$; data sharing same character are not significantly different.

Treatment	STAGE 1: BUD BREAK				STAGE 2: MULTIPLICATION		
	% contamination	% bud break			% contamination	No. of shoots/explant	Final yield/ 100 explants
		10 d	20 d	30 d			
a+ s-	5	24[a]	68[a]	91[a]	0	10[a]	870[a]
a+ s+	10	40[b]	83[b]	125[b]	0	17[b]	1921[b]

The results for clone GC550 (Table 3.8) indicated that both explant types exhibited high levels of contamination at both culture stages (maximum 88%). Bud break percentage was significantly highest with explant type 1 subjected to treatment a- s+ (53% of explants). Multiplication levels with stored and non-stored explants were low for both explant types. The highest yield for stored type 1 explants was obtained with explants subjected to treatment a+ s+ (7.3 shoots per explant and 54 shoots per 100 explants). Multiplication yields for explant type 2 were not significantly different, irrespective of treatments used. However, as contamination hindered yields, bud break percentage were low, and final yields (shoots per 100 explants) were lower when compared to these obtained for TAG14, ZG14 and GU210. Therefore, another set of experiments was performed using type 2 and calcium hypochlorite supplemented into bud break medium in order to decrease the levels of contamination. Results obtained (Table 3.9) indicated a one third decrease in contamination levels and bud break percentage increased by a factor of 3.7 in stage 1. A significantly higher multiplication yield of 10 shoots per explant was obtained with stored explants exposed to calcium hypochlorite at stage 1 than when the calcium hypochlorite treatment was not used. The final yields (shoots per 100 explants)

also significantly increased from 19 to 400 shoots (Table 3.9). It was then concluded that to initiate explants of this clone successfully, explant type 2 is the most suitable and needs to be subjected to treatment a+ s+, after which it should be cultured on bud break medium with 1.0 $g.l^{-1}$ calcium hypochlorite supplement.

Table 3.8: Effect of sterilisation and storage treatments on percentage contamination, percentage bud break, multiplication and final yield per 100 explants of two types of explants of non-fungicide treated GC550. Pre-treatments: a+ = sprayed with 70% (v/v) alcohol, a - = not sprayed, s+ = stored for 48 h in sterile moistened vermiculite in the dark at room temperature and s - = not stored. Explant types: 1 = nodal explant with one half leaf and 2 = stem piece with three nodes. Mean separation within columns ($p \leq 0.05$). n = 20. $^{a-c}$; data sharing same character are not significantly different.

		STAGE 1: BUD BREAK		STAGE 2: MULTIPLICATION		
Explant type	Treatment	% contamination	% bud break	% contamination	No. of shoots/explant	Final yield/ 100 explants
1	a-s-	27	20^{b}	55	5^{ab}	35^{b}
	a-s+	20	53^{c}	75	1.7^{a}	19^{ab}
	a+s-	40	27^{ab}	67	2.7^{a}	15^{ab}
	a+s+	53	27^{ab}	43	7.3^{b}	54^{c}
2	a-s-	47	4.4^{a}	75	8.5^{b}	5^{a}
	a-s+	40	22^{b}	78	4.5^{ab}	14^{ab}
	a+s-	47	8.9^{a}	88	4^{ab}	2^{a}
	a+s+	60	18^{b}	50	4.7^{ab}	19^{ab}

Table 3.9: Effect of sterilisation, storage treatments, calcium hypochlorite (1.0 $g.l^{-1}$), on percentage contamination, percentage bud break, multiplication and final yield per 100 explants of a+ s+ explant type 2 of non-fungicide treated GC550. Pre-treatments involved spraying with 70% (v/v) alcohol: (a+) and storage for 48 h in sterile moistened vermiculite in the dark: (s+) at room temperature. Explant type 2 = stem piece with three nodes. Mean separation within columns ($p \leq 0.05$). n = 20. $^{a-c}$; data sharing same character are not significantly different.

STAGE 1: BUD BREAK			STAGE 2: MULTIPLICATION		
Calcium hpochlorite ($g.l^{-1}$)	% contamination	% bud break	% contamination	No. of shoots/explant	Final yield/ 100 explants
-	60	18^{a}	50	4.7^{a}	19^{a}
1.0	40	67^{b}	0	10^{b}	400^{b}

3.1.3 Studies with TAG5

It was important to test this clone for *in vitro* collection trials because it is among those top-rated by Mondi for disease resistance, growth characteristics on various sites, and a pulp that has consistent fibre quality and guarantees end-user satisfaction (Blakeway, pers. comm[1].).

Results obtained (Table 3.10) indicated that, at both culture stages, contamination levels were low with both explant types (maximum 25%). Explant type 1 subjected to treatment a+ s+ had a significantly high bud break (up to 100%). Multiplication levels obtained with stored type 2 explants were significantly higher (22 and 28 shoots per explant) than either stored or non-stored type 1 explants. Final yields obtained with stored type 2 explants were significantly higher (up to 1624 shoots) than type 1.

[1] F. C. Blakeway, Mondi Forests, Mountain Home Laboratory, Hilton, KZN, South Africa.

In conclusion, for this clone, the best explant for *in vitro* collection is type 2 and most suitable treatment, a+ s+. Results obtained with this clone showed sufficiently high yields and thus the protocol developed was tested on field material.

Table 3.10: Effect of sterilisation and storage treatments on percentage contamination, percentage bud break, multiplication and final yield per 100 explants of two types of explants of fungicide-treated TAG5. Pre-treatments: a + = sprayed with 70% (v/v) alcohol, a - = not sprayed, s+ = stored for 48 h in sterile moistened vermiculite in the dark at room temperature and s - = not stored. Explant types: 1 = nodal explant with one half leaf and 2 = stem piece with three nodes. Mean separation within columns ($p \leq 0.05$). n = 20. $^{a-c}$; data sharing same character are not significantly different.

		STAGE 1: BUD BREAK		STAGE 2: MULTIPLICATION		
Explant type	Treatment	% contamination	% bud break	% contamination	No of shoots/explant	Final yield/ 100 explants
1	a-s-	20	70^{b}	25	2^{a}	84^{a}
	a-s+	10	80^{b}	0	7^{a}	504^{b}
	a+s-	0	60^{ab}	20	4^{a}	192^{ab}
	a+s+	0	100^{c}	10	6^{a}	540^{b}
2	a-s-	0	63^{ab}	0	15^{ab}	945^{bc}
	a-s+	20	73^{b}	0	28^{b}	1624^{c}
	a+s-	20	50^{a}	25	17^{ab}	510^{b}
	a+s+	10	67^{ab}	0	22^{b}	1320^{c}

3.2 Testing the developed protocol: studies with field material

From all the studies discussed above, it can be concluded that the most appropriate storage method while 'transporting' explants is in moistened sterile vermiculite within a closed tissue culture bottle. Explant type 2 subjected to treatment (a+) prior to storage in the field was most suitable for almost all genotypes investigated. Further, for successful culture initiation of the plant material more susceptible to contaminants, the culture medium required supplementating with calcium hypochlorite at 1.0 $g.l^{-1}$ to decrease levels of contamination. Thus, this protocol was employed for field material of pure *E. grandis* clones of ZG14, TAG5 and TAG14. These clones were selected because they were the only plant material available in the field at Kwa-Mbonambi during the time of the study.

The results for ZG14 (Table 3.11) indicated that incorporation of calcium hypochlorite in bud break medium seemed to decrease levels of contamination for both stored and non-stored explants in stage 1, although at stage 2 levels were high (maximum 82%) irrespective of treatments used. A significantly higher bud break percentage was obtained with stored explants (60%) cultured on medium with calcium hypochlorite. However, the presence of calcium hypochlorite in the first culture stage inhibited shoot multiplication in the subsequent culture stage. Nevertheless, calcium hypochlorite facilitated a final yield of up to 264 shoots per 100 explants. In conclusion, when collecting field material of this clone for either storage or immediate culture initiation explants should be treated with alcohol and cultured on bud break medium with a calcium hypochlorite supplement.

Table 3.11: Effect of sterilisation, storage treatments and calcium hypochlorite (1.0 $g.l^{-1}$) on percentage contamination, percentage bud break, multiplication and final yield per 100 type 2 of field plants ZG14. Pre-treatments: a+ = sprayed with 70% (v/v) alcohol, a- = not sprayed, s+ = stored for 48 h in sterile moistened vermiculite in the dark at room temperature and s- = not stored. Explant type 2 = stem piece with three nodes. Mean separation within columns ($p \leq 0.05$). n = 20. $^{a-c}$; data sharing same character are not significantly different.

Treatment	STAGE 1: BUD BREAK			STAGE 2: MULTIPLICATION		
	Calcium hypochlorite ($g.l^{-1}$)	% contamination	% Bud break	% contamination	No. of shoots/explant	Final yield/ 100 explants
a- s-	-	45	30^{a}	82	7^{a}	1.3^{a}
a- s-	1.0	25	50^{b}	53	9^{a}	8^{ab}
a+ s+	-	25	43^{ab}	77	10^{b}	70^{b}
a+ s+	1.0	5	60^{c}	42	8^{a}	264^{c}

Similar results were obtained with TAG5 (Table 3.12) when compared to ZG14 (Table 3.11). Stored explants subjected to calcium hypochlorite at stage 1 had low contamination (10%), but this increased in stage 2 (44%). A significantly high bud break percentage (70% of explants) was obtained with non-stored explants exposed to calcium hypochlorite. A multiplication yield of 10 shoots per explant was obtained with non-stored explants cultured on medium, whether or not calcium hypochlorite was included. Supplementing the medium with calcium hypochlorite for non-stored explants resulted in multiplication yields similar to explants that were not subjected to this treatment. However, non-stored explants subjected to calcium hypochlorite at stage 1 had a significantly higher final yield of 320 shoots when compared with 130 shoots when calcium hypochlorite was omitted. Inclusion of calcium hypochlorite in the media for stored explants (a+ s+) resulted in lower, but reasonable final yield of 202 shoots. Therefore, it was concluded that for field material of TAG5, treatment a+ s+ and explants cultured on medium with 1.0 $g.l^{-1}$ calcium hypochlorite were most suitable for *in vitro* field collection.

Table 3.12: Effect of sterilisation, storage treatments and calcium hypochlorite (1.0 $g.l^{-1}$) on percentage contamination, percentage bud break, multiplication and final yield per 100 type 2 explants of field plants TAG5. Pre-treatments: a+ = sprayed with 70% (v/v) alcohol, a- = not sprayed, s+ = stored for 48 h in sterile moistened vermiculite in the dark at room temperature and s- = not stored. Explant type 2 = stem piece with three nodes. Mean separation within columns ($p \leq 0.05$). n = 20. $^{a-c}$; data sharing same character are not significantly different.

Treatment	STAGE 1: BUD BREAK			STAGE 2: MULTIPLICATION		
	Calcium hypochlorite ($g.l^{-1}$)	% contamination	% Bud break	% contamination	No. of shoots/explant	Final yield/ 100 explants
a- s-	-	30	37^{ab}	50	10^{b}	130^{ab}
a- s-	1.0	15	70^{c}	47	10^{b}	320^{c}
a+ s+	-	50	23^{a}	70	4^{a}	14^{a}
a+ s+	1.0	10	50^{b}	44	9^{b}	202^{b}

The results obtained for TAG14 (Table 3.13) indicated in a similar trend to those obtained for ZG14 and TAG5 (Tables 3.11 and 3.12, respectively). At stage 1, lowest contamination (20%) occurred with stored explants that were subjected to calcium hypochlorite, but contamination increased for all explants in stage 2. The bud break percentages obtained were below 50%, irrespective of the treatments used. The highest values were obtained with both stored and non-stored explants (43 and 45 of explants), cultured on medium with calcium hypochlorite supplement at stage 1, which were significantly different from those where this supplement was omitted. The highest multiplication yield of 14 shoots per explant was obtained with non-stored explants not subjected to calcium hypochlorite, but this was not significantly higher than shoot production by stored explants cultured on medium with calcium hypochlorite supplement. A significantly highest final yield of 160 shoots was obtained with stored explants initially subjected to calcium hypochlorite supplement, probably because of low contamination at stage 1. Results with this clone also indicated that when explant type 2 was subjected to treatment a+ s+ and cultured on calcium hypochlorite-supplemented

medium, decreased contamination levels and greater yields were obtained. Therefore, for all field material investigated in this study, explant type 2 subjected to treatment a+ s+ gave better yields and appeared to be most suitable for *in vitro* field collection. Further, as inclusion of calcium hypochlorite in the medium was associated with a decrease in levels of contamination it should be used for successful culture initiation.

Table 3.13: Effect of sterilisation, storage treatments and calcium hypochlorite (1.0 $g.l^{-1}$) on percentage contamination, percentage bud break, multiplication and final yield per 100 type 2 of field plants TAG14. Pre-treatments: a+ = sprayed with 70% (v/v) alcohol, a- = not sprayed, s+ = stored for 48 h in sterile moistened vermiculite in the dark at room temperature and s- = not stored. Explant type 2 = stem piece with three nodes. Mean separation within columns ($p \leq 0.05$). n = 20. $^{a-c}$; data sharing same character are not significantly different.

Treatment	STAGE 1: BUD BREAK			STAGE 2: MULTIPLICATION		
	Calcium Hypochlorite ($g.l^{-1}$)	% contamination	% Bud break	% contamination	No. of shoots/explant	Final yield/ 100 explants
a- s-	-	60	15^{a}	75	14^{b}	21^{a}
a- s-	1.0	30	43^{b}	57	9^{a}	116^{b}
a+ s+	-	50	20^{a}	50	8^{a}	40^{ab}
a+ s+	1.0	20	45^{b}	63	12^{b}	160^{c}

3.3 Protocol development for *in vitro* collection and storage: Studies on *Trichilia dregeana*

Plant germplasm conservation, collection and distribution are often hindered by spoilage of samples in transit. For purposes of this study, preliminary sterilisation protocols were developed to decrease the rate at which deterioration takes place, by eliminating surface microbial contamination. Suitable transportation conditions were also developed for seed material from the collecting site to the laboratory. Two approaches were investigated. The first involved temporary seed storage in salt solutions [i.e. potassium chloride (KCl), potassium sorbate ($C_6H_7KO_2$) and sodium benzoate (C_6H_5COONa), each at 16 $g.l^{-1}$]. Seeds were transported in these solutions to the laboratory. Seeds were then sampled after 24, 48 and 72 h to test for viability/germination in vermiculite and contamination levels of embryonic axes (and small cotyledon segments) cultured on medium containing MS nutrients, 30 $g.l^{-1}$ sucrose and 10 $g.l^{-1}$ agar (2.2.2). The second approach involved the direct introduction of embryonic axes into culture in the field, on water agar medium containing various combinations of anti-fungal and bactericidal agents (2.3). The most suitable procedure was also evaluated in terms of contamination and ability of axes to germinate and form plantlets. These two approaches were compared in terms of the most feasible operational procedure, giving less contamination and greater yields.

3.3.1 Development of a protocol for short-term storage of *T. dregeana*

The contamination levels observed with *T. dregeana* material (Table 3.14), after temporary storage in potassium chloride and potassium sorbate solutions were relatively low at 24 h (24 and 38% respectively), but as the period of storage increased, contamination levels, although lower after 48 h, had increased considerably by 72 h. Germination percentages (Table 3.14) indicated 100% viability after 24 h of temporary seed storage for all solutions. Germination percentage after 48 h storage had decreased slightly to 96%, for seeds maintained in both potassium chloride and potassium sorbate whereas with sodium benzoate a decrease of 12% was observed. Germinability then

decreased as the period of storage was increased. Potassium sorbate and sodium benzoate showed phytotoxicity effects on seeds after 72 h of storage resulting in germination levels of less than 50%, with 88% of the seeds germinating after 72 h in potassium chloride. In conclusion, temporary pre-storage in potassium chloride and potassium sorbate without any surface sterilisation in the field can be employed for a storage period of 48 h. Potassium chloride appears to be suitable for temporary seed pre-storage under field conditions for up to 72 h. Contamination level also, was significantly lower after 72 h in seeds stored in potassium chloride, compared with those in potassium sorbate.

Table 3.14: Effect of pre-storage on *T. dregeana* seed germination in vermiculite for 2 weeks and embryonic axis contamination after culture on medium containing MS nutrients, 30 $g.l^{-1}$ sucrose and 10 $g.l^{-1}$ agar for 30 days. (see 2.2.1- *Batch 1*). Mean separation within columns ($p \leq 0.05$). n=30. $^{a-c}$: data sharing same character are not significantly different.

		% Germination			% Contamination		
	Time (h)	24	48	72	24	48	72
Storage condition	KCl	100^{a}	96^{b}	88^{b}	24^{a}	30^{b}	46^{a}
	$C_6H_7KO_2$	100^{a}	96^{b}	40^{a}	38^{ab}	21^{a}	68^{b}
	C_6H_5COONa	100^{a}	88^{a}	36^{a}	50^{b}	36^{b}	52^{ab}

Seeds that were surface sterilized in the field with 1% sodium hypochlorite for 20 min, rinsed in distilled water and soaked in a combination of fungicides for 2 min, then rinsed again in distilled water before being transferred into potassium chloride showed a maximum germination (100%) at 72 h and contamination levels below 50% (2.2.1 Batch 3; Table 3.15). Although contamination levels were significantly lower for seeds after 72 h in potassium sorbate and sodium benzoate, their viability was seriously compromised,

supporting the previous observation that these solutions have a time-related phytotoxic effect.

Much lower contamination levels were observed (maximum 20%) for each storage solution used after 72 h for seeds that were surface sterilised in the field with 1% (v/v) sodium hypochlorite for 20 min and without any rinsing, transferred into the temporary storage solutions (2.2.1 Batch 2; Table 3.16). However, as shown for seed batches 1 and 3, at the concentration of potassium sorbate and sodium benzoate used, both solutions have phytotoxic effects after 72 h.

In conclusion, surface sterilisation of seeds in the field before storage in salt solutions can be employed, and the most suitable procedure used was surface sterilization with 1% (v/v) sodium hypochlorite for 20 min, followed by transfer of seeds without any rinsing into potassium chloride. Seeds with the aril removed, surface sterilised alone and stored in KCl solution (16 $g.l^{-1}$) offers the advantage of simplicity and lessens the materials necessary for *in vitro* field collecting. Seedlings in vermiculite grown from seeds treated by this procedure, are shown in (Fig. 3.2).

Table 3.15: Effect of surface sterilisation, fungicide treatment and pre-storage on *T. dregeana* seed germination in vermiculite for 2 weeks and embryonic axis contamination after culture on medium containing MS nutrients, 30 $g.l^{-1}$ sucrose and 10 $g.l^{-1}$ agar for 30 days. (see 2.2.1- *Batch 3*). Mean separation within columns ($p \leq 0.05$). n=30. $^{a-c}$: data sharing same character are not significantly different.

		% Germination			% Contamination		
	Time (h)	24	48	72	24	48	72
Storage condition	KCl	100^{a}	100^{b}	100^{b}	47^{a}	40^{ab}	40^{b}
	$C_6H_7KO_2$	100^{a}	93^{b}	53^{a}	40^{a}	57^{b}	20^{a}
	C_6H_5COONa	100^{a}	80^{a}	71^{ab}	40^{a}	20^{a}	13^{a}

Table 3.16: Effect of surface sterilisation alone before pre-storage, on *T. dregeana* seed germination in vermiculite for 2 weeks and embryonic axis contamination after culture on medium containing MS nutrients, 30 $g.l^{-1}$ sucrose and 10 $g.l^{-1}$ agar for 30 days (see 2.2.1-*Batch 2*). Mean separation within columns ($p \leq 0.05$). n=30. $^{a-b}$: data sharing same character are not significantly different.

		% Germination			% Contamination		
	Time (h)	24	48	72	24	48	72
Storage condition	KCl	100^a	100^a	100^b	40^b	27^b	20^b
	$C_6H_7KO_2$	100^a	100^a	71^a	20^a	13^a	7^a
	C_6H_5COONa	100^a	86^a	67^a	20^a	13^a	0^a

Figure 3.1: Seedlings of *T. dregeana* in vermiculite. Seeds had been treated as detailed in (2.2.1 , Batches 1, 2 and 3) [bar = 3.7 mm].

3.3.2 Development of a protocol for the collection and culture of embryonic axis of *Trichilia dregeana* in the field

Because of fairly high levels of both fungal and bacterial contaminants (not identified) (Tables 3.14, 3.15 and 3.16), a different approach for *T. dregeana* germplasm collection in the field was employed in an attempt to circumvent this problem. Collection and surface sterilisation are as described in section 2.3. Embryonic axis were then introduced in the field into sterile tubes containing pre-prepared semi-solid water agar, which had been supplemented with various anti-fungal/bactericidal agents (Table 2.3). These were transported in this state and maintained in culture for a period of 10 days, after/which contamination was recorded. Embryonic axes that did not show contamination were then transferred onto germination medium containing MS nutrients, 30 $g.l^{-1}$ sucrose and 10 $g.l^{-1}$ agar for germination and further contamination assessments.

The results of this investigation (Table 3.17) at stage 1 revealed that those axes that were cultured on a non-supplemented water agar exhibited a contamination percentage of 33%. Impact alone, the mixture of Impact and Previcur and that of Kanamycin, and the Impact and Previcur combination reduced both fungal and bacterial contamination to zero in stage 1.

Results for Benomyl-supplemented medium showed a maximum of 3% contamination but when axes where transferred onto germination medium, only 15% were viable. Thus, usage of Benomyl at 1.0 $g.l^{-1}$ was lethal to most of the embryonic axes. When embryonic axes from all other treatments in stage 1 were transferred to germination medium, 100% germination was obtained (Fig. 3.3) with an acceptably low contamination levels (15 - 22%).

The best combination of anti-microbial agents used was Kanamycin, Impact and Previcur at concentrations indicated (Table 3.17). When comparing the two procedures employed (3.3.1 and 3.3.2); direct inoculation of embryonic axes onto media with anti-microbial agents in the field emerged as the better method both in terms of lower contamination and

the germination percentages obtained. Furthermore, this method can be considered as more efficient, because embryo development can be monitored without any further manipulations until subculture onto medium containing MS nutrients, 30 $g.l^{-1}$ sucrose and 10 $g.l^{-1}$ agar germination-promoting medium, and significantly, less space is required by samples while in transit.

Table 3.17: Effect of surface sterilisation and anti-microbial agents in the medium used for culture initiation in the field, on embryonic axes of *T. dregeana*. Culture was on water agar for 10 d followed by subculture of uncontaminated axes only, on to a medium containing MS nutrients, 30 $g.l^{-1}$ sucrose and 10 $g.l^{-1}$ agar for 2 months. Mean separation within columns ($p \leq 0.05$). n=50. $^{a-b}$: data sharing same character are not significantly different.

STAGE 1 Culture initiation on water agar in the field			STAGE 2 Growth on MS medium in the lab.	
Compound	Concentration	% Contamination.	% Germination	% Contamination
None	-	33^{b}	100^{b}	37^{b}
Impact	0.2 $ml.l^{-1}$	0^{a}	100^{b}	22^{ab}
Previcur	2.5 $ml.l^{-1}$	7^{a}	100^{b}	20^{ab}
Impact and previcur	0.2 $ml.l^{-1}$ and 2.5 $ml.l^{-1}$	0^{a}	100^{b}	20^{ab}
Benomyl	1.0 $g.l^{-1}$	3^{a}	15^{a}	0^{a}
Kanamycin	0.64 $g.l^{-1}$	3^{a}	100^{b}	18^{ab}
Kanamycin, impact and previcur	0.64 $g.l^{-1}$, 0.2 $ml.l^{-1}$ and 2.5 $ml.l^{-1}$	0^{a}	100^{b}	15^{ab}

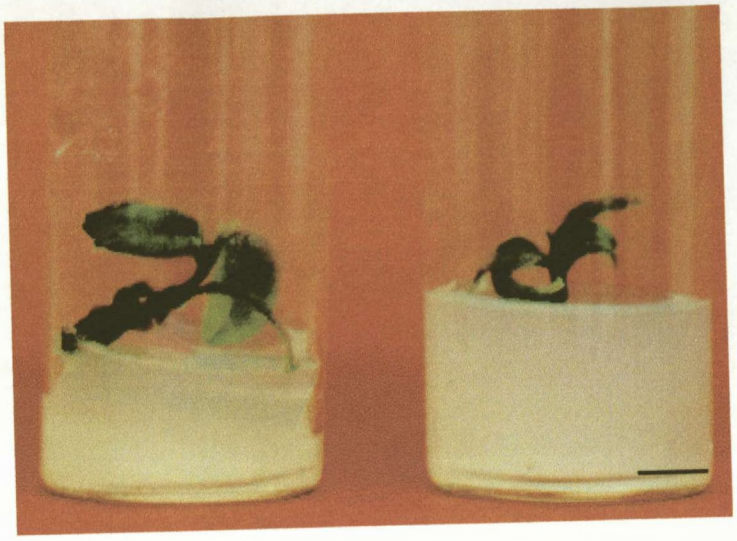

Figure 3.2: Germinated plants from embryonic axes after 2 months on medium containing MS nutrients, 30 g.l^{-1} sucrose and 10 g.l^{-1} agar [bar = 4 mm].

4. DISCUSSION

4.1 Effects of sterilisation and storage on contamination, bud break, multiplication and final yield of *Eucalyptus* explants

4.1.1 *In vitro* collection of explants

The collection of living plant materials for storage or culture *ex situ* is a potentially powerful tool for collecting and preserving plant diversity (Pence, 1996). *In vitro* collecting techniques are effective for a range of species, and have been developed to facilitate the timing of collection and to reduce bulk for transport, while maintaining viability of tissue during transport. This approach provides the means for distribution of uncontaminated germplasm (Ashmore, 1997).

For purposes of this study, two simulations of conditions for 48 h of transport were investigated, to establish a suitable collecting procedure (Figures 2.1 and 2.2). Regardless of surface sterilisation treatment used, explants stored in sterile moistened vermiculite enclosed in a tissue culture bottle exhibited no contamination and had greater than 50% bud break, in contrast to explants transported in cling wrap, where contamination was high and bud break levels low (Table 3.1). Moreover, it was also noted that after explants were stored for 48 h in cling wrap, they appeared dry and showed brown patches, whereas explants in vermiculite bottles were moist and retained the original pigmentation with no discoloured areas. According to Pence (1996), an *in vitro* storage/collection method employed at the collecting site must prevent the tissue from browning and stimulate growth, either through bud outgrowth (when preformed buds are collected) or by regeneration, when non-meristematic tissue is collected. Thus it was concluded that tissue culture bottles containing sterile moistened vermiculite provided a suitable *in vitro* collection and storage method for the explants. The literature consulted does not include any parallel work for *in vitro* collection of *Eucalyptus* species. However, simple methods of *in vitro* collecting for other species have been developed (e.g. *Cocos nucifera*, *Musa*, *Coffea* and *Citrus* spp.). For those species, stem nodal

cuttings and leaf discs were used; explants of some of the species were surface sterilised with 70% ethanol and others with a calcium hypochlorite solution (70% active chlorine) and before inoculation into semi-solid medium containing anti-microbial agents, in the field (Withers, 1987, Yidana *et al.*, 1987, Assy-Bah *et al.*, 2000). Semi-solid rather than liquid medium provides greater physical stability. Semi-solid medium was not used in this study, as sterile vermiculite contained in bottles was found to be a convenient and suitable alternative. Alfreen-Zobayed (2000) reported that plantlets grown on mixtures containing various proportions of vermiculite with 30% paper pulp, showed survival percentages ranging from 90% to 100% whereas the survival percentage of those cultured on agar was only 73% for micropropagation of photoautrophic sweet potato plantlets. This is in agreement with the present findings that bottles containing sterile vermiculite were most suitable, economical, and provided a simple and secure storage condition during the collection operation.

4.1.2 Production of *in vitro* shoot explants

Sterile shoot cultures were successfully generated from sprouted branches (approximately 100 mm in length) of *E. grandis* species and hybrids. From the trial experiments on both fungicide and non-fungicide treated plant material, the highest shoot yield per stored explant was 16 for type 1 and 30 for type 2. It is also significant that similar shoot yields have been reported in the literature for *Eucalyptus* species and hybrids but without storage [e.g. per explant: 5 – 10 shoots for *E. citriodora* (Gupta *et al.*, 1981); 11 shoots for *E. globulus* (Bennett *et al.*, 1992; 1994) and 10 shoots for *E. grandis x urophylla* (Yang *et al.*, 1995)]. The number of shoots per explant obtained with field material was also adequate (up to a maximum of 14 shoots per explant with type 2). Losses of explants as culture stages progressed were due to contamination rather than because of any phytotoxic effects of the sterilisation treatments employed, as discussed in the next section.

4.1.3 Losses by contamination

The first concern in developing *in vitro* collecting techniques is to limit contamination in the cultures (McComb and Bennett, 1986; Pence, 1996). Contamination can be a problem in tissue culture systems in the best circumstances, but with field material the greatest limitations of micropropagation of eucalypts are the problems associated with sterilisation (McComb and Bennett, 1986). Those authors pointed out that it is difficult to obtain 'clean' viable material for production of *in vitro* cultures if field-grown explants are used. Other results with *Eucalyptus* species have also proved that use of explants from the field-grown material is often associated with intensive contamination (Watt *et al.*, 1995; 1996).

In this study, *in vitro* collecting methods were developed in order to minimise some of the contamination problems associated with field-collected explants. The protocol was developed using parent plants treated or not treated with fungicides, stored type 1 and 2 explants (ZG14 and TAG5) sprayed with alcohol obtained from greenhouse plants (treated with fungicides) were successfully regenerated and only a few explants $\leq$ 10% were lost due to contamination (Tables 3.2 and 3.10). In contrast, explants from non-fungicide-treated specimen grown outside the greenhouse (which mimicked the field conditions) had relatively high contamination levels as reported by e.g. Warrag *et al.* (1990) and le Roux and van Staden (1991*b*). Explants of TAG14, GU210 and GC550 (Tables 3.5, 3.6 and 3.8) showed low contamination levels at early culture stages, but contamination percentages progressively increased thereafter. Similar results have been reported with *in vitro* culture of plant tissue, in which growth of microbial contaminants is temporarily suppressed, but becomes evident after several subcultures (De Fossard *et al.*, 1977; Gupta *et al.*, 1981; Dodds and Roberts, 1985; le Roux and van Staden, 1991; Warren, 1991; Gamborg and Phillips, 1995). The present results indicated that further strategies had to be employed to deal with field material.

Studies with ZG14 (not treated with fungicides) (Table 3.3) were extremely difficult because of high levels of contamination (up to 100%). In such cases, the bud break stage

was selected for the testing of the effect of anti-microbial agents (Benomyl and calcium hypochlorite). Benomyl was incorporated into the bud break medium at the same concentrations as used by Watt *et al.* for *Eucalyptus* explants (1996) while calcium hypochlorite was included at concentrations of 0.5 and 1.0 $g.l^{-1}$. Yanagawa (2000) reported that incorporating active chlorine at 0.005 – 0.01% directly into the medium eliminated contamination problems when orchid seedlings were grown *in vitro*. That author also stated that this procedure was effective for transplanting plantlets and calli of several ornamental plants cultured *in vitro* under non-sterile conditions. The percentages of active chlorine equivalent to the 0.5 and 1.0 $g.l^{-1}$ calcium hypochlorite presently used, were 0.05% and 0.1%.

Benlate is the commercial name used by the du Pont Corporation for methyl-butylcarbanamoyl-2-benzimidazole, while the local product is marketed by Sanachem, South Africa, as Benomyl. It is a systemic fungicide used in forestry practice, principally in nurseries, to control fungal infestations (Carmargo *et al.*, 2000), Hong (1976) associated the use of Benlate with shoot vigour promotion in *Vitis vinifera,* but for *Pinus caribeceae* seedlings, mycorrhiza development was repressed. Thurston *et al.* (1979) reported that growth of orchids was best when they were micropropagated on media containing Benlate. Calcium hypochlorite, generally used as diluted household detergent, is used as a surface sterilant to eliminate superficial contaminants (George, 1993; Abdel-Mallek *et al.*, 1995; Koziara, 2000).

Neither of the tested Benomyl concentrations (0.5 and 1.0 $g.l^{-1}$) appeared to have direct detrimental effects on the *Eucalyptus* explants, nor was bud break inhibited (Table 3.4). These findings contradict those recording phytotoxic effects of 0.5 and 1.0 $mg.l^{-1}$ Benlate by Carmago *et al.* (2000) and 0.5 and 1.0 $g.l^{-1}$ Benomyl by Watt *et al.* (1996) on explants. A possible reason for these contradictory findings may be that bigger, more mature explants were used in the present study: this type of material is thought to be less susceptible to possible fungicide toxicity (Reed and Tanprasert, 1995). The period of exposure and the culture stage could also have negative impacts on successful regeneration of shoots; the period of exposure of the explants to Benomyl in this

investigation was for ten days during bud break, while Watt *et al.* (1996) reported phytotoxic effects after five weeks of exposure in the multiplication stage.

Stored explants presently exposed to calcium hypochlorite (1.0 $g.l^{-1}$) at the bud break stage, exhibited no contamination while 33% of those subjected to Benomyl did. Yanagawa (2000) obtained similar results for calli and explants of several ornamental plants *in vitro* on medium which incorporated calcium hypochlorite. In some of the present cases, however, contamination was low at the first culture stage (maximum of 5%) but increased at the second culture stage (up to a maximum of 82%) (Tables 3.11 to 3.13). The effect of calcium hypochlorite at stage 2 on contamination and multiplication yields was unfortunately not tested, and it is imperative that future work should ascertain whether later-manifested contamination might be diminished and/or whether there would be phytotoxic effects on the explants. Explants will also need to be transferred onto an appropriate medium to assess rooting potential of the survivors.

The frequently-encountered problem of high levels of contamination occurring at the multiplication stage, might be eliminated by use of systemic fungicides being taken up into the tissue (rather than only in contact with the surface); this is a further possibility which should be investigated in future studies. Carmago *et al.* (2000) reported that for *E. grandis* explants cultured on medium with Nizoral® (cetoconazol) fungicide produced a large number of shoots per explant and those grown in medium supplemented with Nystatin (mycostatin) showed the best level of root induction (67%) and low contamination levels.

4.1.4 Applications of the protocol

It is implicit that successful establishment of this protocol would lead to a general procedure for *in vitro* collection of various *Eucalyptus* species. The *in vitro* collection method employed in this study appears to have potential in terms of the adequate multiplication yields obtained. Explants collected were surface sterilised with alcohol

only, enclosed into bottles with sterile vermiculite and then transported into the laboratory for later use. The multiplication yields obtained with *Eucalyptus* clones (TAG14, ZG14 and TAG5) and the hybrids (GC550 and GU210) were adequate and similar to those reported in the literature for non-stored explants as discussed in 4.1.2. Only small amounts of the parent plant were harvested (i.e. a stem segment with three nodes), allowing the rest of the plant in the hedge to remain undisturbed. Collectors in remote areas could also use this procedure to avoid having to return often to these sites. However, laboratories receiving such material should take precautions before initiating tissue culture systems because of the unknown endogenous contamination status. Some of the material used in this study exhibited higher contamination levels than others: ZG14>TAG14>TAG5. Under such circumstances, addition of calcium hypochlorite at 1.0 $g.l^{-1}$ to the medium is advised as a precaution.

Only three *Eucalyptus grandis* clones from the field were tested, which represents less than 1% of the total clones used by Mondi Forests. From the results it was evident that variations occurred from clone to clone with regard to both final yields and contamination. Therefore more clones need to be tested to confirm the value of the protocol.

4.2 *Trichilia* embryonic axis development and contamination *in vitro*

In vitro field collection protocols need to be devised to ensure distribution of healthy plant germplasm that is free from pathogens (Ashmore, 1997). In this investigation, two approaches to seed or seed-derived germplasm collection in the field were investigated. The first involved storage of seeds in salt solutions, while in the second direct introduction of embryonic axes into culture in the field was carried out. In the first investigation, unsatisfactorily high levels of contamination were obtained despite the surface sterilisation of embryonic axes after being isolated and the use of supposedly sterile inoculation conditions in the laboratory. It was also observed that the longer the period of seed storage in the salt solutions, the higher the levels of contamination. In

contrast, employment of the second approach resulted in low contamination levels at both culture stages.

Outdoors axis excision and culturing with such precautions as could be taken, and the use of sterile medium with anti-microbial supplements, gave a contamination level not exceeding 7% in stage 1 (Table 3.17). This is highly satisfactory when compared to embryonic axes cultured on non-supplemented medium, where a 33% level of contamination occurred. To conclude, the two approaches described can be used for germplasm collection of *T. dregeana* zygotic germplasm. If the collecting site is near the *in vitro* laboratory, the first approach of seed sampling and storage is more suitable, as it is simple and quick. For longer storage, the second approach of direct inoculation into culture emerged as superior in terms of the low contamination. Furthermore, period of storage for up to 10 days without increase of the levels of contamination or disruption of embryo development was presently achieved, and it is probable that even longer-term temporary storage for transport might be possible. Benomyl test trials on *T. dregeana* with 1.0 $g.l^{-1}$ incorporated into the medium, resulted in low contamination levels but showed phytotoxic effects on the embryonic axes; lower concentration levels of Benomyl are recommended for further investigation.

CONCLUSIONS

The objectives of this study were to establish suitable *in vitro* field collection and transportation techniques for vegetative material of *Eucalyptus* species and embryo of *T. dregeana*. It is evident from the results reported that both vegetative explants and seed material can be collected using the *in vitro* techniques developed in this study. This work can serve as the basis for future developments on and applications of *in vitro* collection strategies for *Eucalyptus* and other woody species, and species which have recalcitrant seeds.

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