DEVELOPMENT OF EXPLANTS POTENTIALLY SUITABLE FOR CRYOPRESERVATION OF THE RECALCITRANT-SEEDED SPECIES, *THEOBROMA CACAO* L. AND *BARRINGTONIA RACEMOSA* (L.) ROXB.

by

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ABSTRACT

The two species investigated in this study were *Theobroma cacao* and *Barringtonia racemosa*. *Theobroma cacao* has worldwide economic importance, as cocoa (the main ingredient in chocolate) is produced from the seeds of this tree; while *B. racemosa* has several applications in herbal medicine. The seeds of both *T. cacao* and *B. racemosa* are highly recalcitrant and therefore not amenable to storage for any significant periods. The long-term conservation of the germplasm of these species may only be feasible via cryopreservation. The aims of the present study were to: 1) optimize *in vitro* regeneration protocols for different types of explants that have the potential to be cryopreserved while maintaining the genetic integrity of these two species; and 2) develop cryopreservation protocols for selected explants.

For *T. cacao*, protocols were established for bud-break and multiplication for both *in vitro*- and greenhouse-derived nodal explants, as well as a rooting medium for shoots derived from axillary buds. Parameters investigated towards the cryopreservation of axillary shoots, from greenhouse nodal segments, and nodal segments from *in vitro* plantlets, included the size of the explant and pre-treatments for cryopreservation. Nodal segments (6 - 7 mm) and axillary shoots (2 - 4 mm) needed to be soaked in 0.5% (w/v) ascorbic acid for 10 min to minimise phenolic production and subsequent tissue death, and surface-sterilized by soaking in 1% $Ca(OCl)_2$ solution for 5 min to reduce microbial contamination. Subsequent cryopreservation attempts involved only *in vitro* nodal segments because of the lack of success in achieving elongation of excised axillary buds. Vitrification and slow freezing methods, with or without the application of cryoprotectants, did not achieve successful cryopreservation. Attempts to establish a protocol for producing somatic embryos, as an alternate to axillary shoots and *in vitro* nodal segments, resulted in the production of globular embryogenic callus for both leaf and cotyledon explants. Cryopreservation of these explants was not investigated in the scope of this study.

The study on *B. racemosa* focused on the development of a somatic embryogenesis protocol. Segments of embryonic axes produced globular-stage embryos when placed on MS medium supplemented with 30 g l^{-1} sucrose, 1.0 g l^{-1} casein hydrolysate, 2.0 mg

l^{-1} 2,4-D, 0.1 mg l^{-1} BAP and 8.0 g l^{-1} agar. Various strategies were employed to obtain embryo germination, which included 1) different time intervals on callus initiation medium; 2) the use of different auxins (IAA, NAA and 2,4-D) in combination with the cytokinins BAP and kinetin; 3) desiccation and 4) cold treatments. Although somatic embryo germination was not achieved, globular embryos proceeded with development to the cotyledonary stage when cold-treated for 8 h at 4˚C.

This study provides some fundamental bases for further investigation towards achieving long-term conservation for both *T. cacao* and *B. racemosa*. However, the use of meristems as explants for cryopreservation is suggested to be the way forward for the cryopreservation of both species.

PREFACE

The experimental work described in this dissertation was carried out in the School of Biological and Conservation Sciences, University of KwaZulu-Natal, Durban from January 2006 to December 2007, under the supervision of Professor P. Berjak, Dr. J. Kioko and Professor N.W. Pammenter.

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

DECLARATION 1 - PLAGIARISM

I, Prabashni Naidoo declare that

1. The research reported in this thesis, except where otherwise indicated, is my original research.

2. This thesis has not been submitted for any degree or examination at any other university.

3. This thesis does not contain other persons' data, pictures, graphs or other information, unless specifically acknowledged as being sourced from other persons.

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(August, 2008)

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LIST OF ABBREVIATIONS

ABA	abscisic acid
BAP	6-Benzylaminopurine
˚C	degrees Celsius
$Ca(OCl)_2$	calcium hypochlorite
CaMg	calcium magnesium
CH	casein hydrolysate
2,4-D	2,4-dichlorophenoxyacetic acid
d	day
DKW	Driver and Kuniyuki (1984) nutrient formulation
dmb	dry mass basis
DMI	demethylation-inhibitor fungicide
DMSO	dimethylsulphoxide
EtOH	ethanol
g	gram
GA_3	giberellic acid
h	hour
HCl	hydrochloric acid
IAA	indole-3-acetic acid
IEDCs	induced embryogenically determined cells
2-iP	isopentenyl adenine
l	litre
LN	liquid nitrogen
M	Molar
m	metre
min	minute
ml	millilitre
mm	millimeter
MS	Murashige and Skoog (1962) nutrient formulation
NAA	1-naphthalene acetic acid

NaOH	sodium hydroxide
NT	not tested
%	percent/percentage
PEDCs	pre-embryogenic determined cells
PGR	plant growth regulator
pH	hydrogen ion content
PPFD	photosynthetic photon flux density
RITA	Recipient of Automated Temporary Immersion system
s	second
TDZ	thidiazuron
μ	micro

CHAPTER 1: INTRODUCTION

1. Conservation of plant genetic resources

Plant germplasm conservation is the management of plant resources to preserve current levels of diversity and to avoid population and taxonomic extinctions (Maunder, 2001). The existence of almost all life forms is, directly or indirectly influenced by the existence of green plants (Staritsky, 1997). The unrestrained exploitation of plant biodiversity and genetic resources has become a global concern over recent years (Razdan and Cocking, 1997; Takagi, 2000; Phartyal, 2002). Plant species from temperate, but more so from tropical, areas are being threatened by biotic and abiotic factors resulting in an increased biological vulnerability of species to extinction (Phartyal *et al*., 2002). The increasing human population, industrialization and other measures of economic growth are disturbing ecosystems worldwide (Razdan and Cocking, 1997), contributing to the loss of biodiversity. In addition, the clearing of forests for agricultural land has caused a depletion of naturally occurring plant genetic resources. Schemske *et al*. (1994) estimated that 25% of the 250, 000 vascular plant species could be extinct within the next 50 years**.**

The International Plant Genetic Resources Institute, IPGRI (now Biodiversity International), plays a major part in plant conservation by offering support for collection as well as conservation of plant germplasm globally (IBPGR, 1984). Thus far, conservation efforts are being made with the aim that by 2010, 60% of the world's species should be conserved *ex situ*. In order to meet this goal suitable management and conservation practices need to be established. It is essential that methods employed for germplasm conservation meet two basic criteria i.e. minimal maintenance and genetic integrity of the stored material (Razdan and Cocking, 1997). There are two types of germplasm conservation: *in situ* and *ex situ*.

1.1. *In situ* Conservation

In situ conservation refers to the conservation of ecosystems and natural habitats, and the maintenance and recovery of viable populations of species in their natural surroundings and, in the case of domesticated or cultivated species, in the surroundings where they have developed their distinctive properties (UNEP, 1992). *In situ*

conservation approaches include genetic reserves on farms and gardens (Maxted *et al*., 1997).

1.2 *Ex situ* Conservation

Ex situ conservation involves the maintenance of the plant outside its natural environment, which can be achieved by acquiring the germplasm and keeping it as an active or base collection. Active collections include plants maintained in field gene banks or botanical gardens, seeds in storage, and *in vitro* plantlets i.e. minimal growth storage (Withers, 1988; Withers, 1989; Staritsky, 1997; Engelmann, 1997). The base collection of plant material *ex situ* involves a process called cryopreservation.

1.2.1 Active collections

Growing plants in field gene banks allows for maintenance of genetic stability within a population but is also associated with high risks (Withers, 1988; Engelmann, 1997). Maintenance costs, a limited amount of genetic variation that can be stored and the vulnerability to natural and human disasters make this method often unfavourable. In the light of this, the most common method of plant conservation involves seed storage (Engelmann, 1997). This method is not only inexpensive, but also relatively simple. However, seed storage cannot be applied uniformly to all species because different plant species produce seeds that are not only morphologically different but also differ physiologically (Berjak and Pammenter, 2004).

Seeds may be broadly classified into three categories, orthodox, intermediate and recalcitrant, depending on the post-harvest properties that determine seed behaviour. Orthodox seeds are those which tolerate extreme degrees of dehydration and maintain high viability when stored at a dehydrated state for long periods at a low temperature (Roberts, 1973). Underlying this are developmental phenomena, particularly maturation drying on the parent plant and being shed dry and quiescent, or if shed wet, the ability to withstand being dried to low water contents without the loss of viability (Roberts, 1973). Such seeds are characterised by desiccation tolerance mechanisms that are acquired relatively early during their pre-shedding development. A suite of mechanisms has been recognized and implicated in the acquisition and maintenance of desiccation

tolerance, including the efficient operation of antioxidant systems; implication of putatively protective molecules, e.g. late embryogenic accumulating/abundant proteins (LEAs) and sugars; and the ability of the cells to repair upon re-hydration (Pammenter and Berjak, 1999; Berjak and Pammenter, 2007).

Intermediate seeds display behaviour between the extremes of desiccation tolerance and desiccation sensitivity. These seeds can tolerate drying but not to levels achievable by orthodox seeds and may be chilling sensitive even in the dehydrated state (Ellis *et al*., 1990; Hong and Ellis, 1998). At the other extreme, there are seeds that lose viability if dried below a relatively high water content; the descriptor coined by Roberts (1973) for these seeds is 'recalcitrant'. Recalcitrant seeds have high water contents and are metabolically active at shedding. Such seeds proceed into germination when stored at the shedding water content, and therefore are amenable only to short-term storage (Berjak *et al*., 1989; Berjak and Pammenter, 2004).

In vitro methods are employed to conserve genetic resources of plant species that cannot be stored as seeds and for which methods of germplasm preservation other than maintenance in the field are not available (Mix-Wagner and Schumacher, 2003). These are recalcitrant-seed producing species and species that are generally vegetatively propagated, such as banana and cassava. The main principle of *in vitro* conservation is maintenance of genetic and physiological characteristics of the preserved material (Kartha, 1987; Withers, 1988; Razdan and Cocking, 1997).

Plant tissue culture, the fundamental component of the *in vitro* method, allows tissues or organs to be excised from the parent plant, surface-sterilised and transferred onto an *in vitro* growth medium, which can be manipulated in various ways to achieve the desired result, ranging from an organised culture to a mass of undifferentiated cells termed callus (George, 1993). The process of tissue culture owes its origin to Haberlandt (1902) who discovered the totipotent ability of plant cells. All living cells containing a normal complement of chromosomes should be capable of regenerating an entire plant (Thorpe, 1990). Plant regeneration through *in vitro* culture can be accomplished via two

developmental pathways: 1). organogenesis and 2). somatic embryogenesis (Reinert and Bajaj, 1977; Bhojwani, 1990; Razdan, 1993; Wilhelm, 2003).

Organogenesis is the formation and outgrowth of organs from explants cultured on media (Narayanaswamy, 1977; Tran Thanh Van and Trinh, 1990). The organogenic process begins with changes in a single or a small group of parenchyma cells, which then divide to produce a globular mass of cells or meristemoid, which is plastic and can give rise to either a shoot or root primordium (Hicks, 1980; Thorpe, 1990). The organs are physically attached to the tissue of origin and are unipolar, i.e. have either a shoot or root meristem (Tran Thanh Van and Trinh, 1990). For most tree species the widely used procedures are either direct organogenesis by promoting pre-formed axillary buds to develop further (most commonly used system in tissue propagation) or indirect organogenesis, achieved by inducing adventitious shoot proliferation (Wilhelm, 2003). Micropropagation via organogenesis follows the order of culture initiation, shoot multiplication, rooting, and acclimatization of plantlets (Wilhelm, 2003). The main purpose of micropropagation is the rapid, clonal multiplication of genotypes (Debergh *et al*., 1990; Smith and Drew, 1990).

Axillary bud culture is achieved by placing nodal segments from the desired plant onto a suitable culture medium, under specific growth conditions, to enhance the production of axillary shoots. The shoots are subcultured onto a multiplication medium, normally including a cytokinin, and the process repeated until a large quantity of plants is obtained, all with the same genetic composition (Hussey, 1983; Smith and Drew, 1990). These shoots are subsequently rooted, usually following the incorporation of an auxin in the culture medium and acclimatized under usually humid greenhouse conditions.

The different phases of the organogenic pathway can be difficult to achieve in some species. The use of growth regulators plays an important role in the morphogenetic responses of the explants during the different phases. Normally the basal medium for culture initiation is of low salt concentrations, especially nitrate and ammonium e.g. WPM (Woody Plant Medium, Lloyd and McCown, 1981). High cytokinin levels are frequently used to force the flushing of the axillary buds, in some instances giving rise

to new shoots (Wilhelm, 2003). These shoots are normally multiplied by dividing them into apical and nodal segments. The reliability of an axillary bud system is proven by the commercial successes gained with different varieties of ornamentals and other angiosperms (Debergh *et al*., 1990)

Somatic embryogenesis is the formation of embryo-like structures arising from somatic cells (Terzi and Loschiavo, 1990; Jun-Yan *et al*., 2000). The first record of embryogenesis dates back to 1958 when Steward and Reinert independently discovered some embryo-like structures while experimenting with the tissues of carrot (*Daucus carota* L.) (Steward *et al*., 1958). Somatic cells develop through the stages of embryogeny to give rise to whole plants without the fusion of gametes (Merkle *et al*., 1990). These structures are bipolar (i.e. have both shoot and root meristems) and have no physical attachment to the maternal tissue/explant of origin (Terzi and Loschiavo, 1990; Thorpe, 1990). Somatic embryogenesis normally encompasses the phases of initiation, multiplication, maturation and germination (Wilhelm, 2003).

The ease with which somatic embryos can form depends largely on the type of explant cultured. Some cells, depending on their origin, have a greater capacity to form somatic embryos compared with others. The capacity of embryogenesis is measured as the epigenetic "distance" of explant cells from the embryogenic state (Merkle *et al*., 1990). Certain plant cells can give rise to somatic embryos directly, these being said to be predetermined (George, 1993; Merkle, 1990), determined cells having undergone an irreversible commitment to a specific morphogenesis pathway (Carman, 1990). There are two types of cell lines that can be identified by the ability to form embryos. These are "pre-embryogenic determined cells" (PEDCs) and "induced embryogenic determined cells" (IEDCs) (Evans *et al*., 1981). Tissues that are uncommitted, IEDCs, to an embryogenic pathway can form embryos directly by exposure to an auxin. Embryogenesis can also be induced via other stimuli such as an electric field, changes in the pH of the culture medium or the incorporation of other regulants, particularly cytokinins to the medium (George, 1996). However, the process of embryogenesis is usually considered to be controlled by auxin either that are produced endogenously within cultured tissues or are supplied from the culture medium (George, 1996).

Once the process of initiation is complete, the multiplication cycle proceeds via secondary embryogenesis. Secondary embryogenesis relies on the incorporation of various growth regulators at low concentrations in some cases, while the addition of plant growth regulators may not be necessary in the other instances (Wilhelm, 2003). The embryo maturation phase is accompanied by major changes in embryonic development, usually organ expansion and the accumulation of nutrient reserves. Different types of stress, such as the use of osmotic compounds, and the application of ABA, desiccation or chilling treatments have been investigated as switches for accumulation of storage products (Wilhelm, 2003). In most cases, explants are transferred to a medium with very low concentrations of auxin or medium devoid of any growth regulators to facilitate embryo maturation (George, 1993). Post maturation, the embryonic phase is terminated by germination that is characterised by the mobilisation of carbohydrate, lipid and protein reserves to enable root and shoot growth. Somatic embryogenesis and organogenesis can occur either directly, structures originating directly from the explant or indirectly, structures originate from an intervening callus stage (Terzi and Loschiavo, 1990).

The somatic embryo propagation system is regarded as the system of choice for mass propagation of superior forest tree genotypes and as a system for genetic improvement (Merkle *et al.*, 1990; Bajaj, 1995; Wilhelm, 2003). The advantages include high multiplication rates, the potential for scale-up in liquid culture with the use of bioreactors and for direct delivery to the greenhouse or field as artificial seeds. Bioreactors may be used as tools to probe stimulus-response in plant cultures and, at the commercial level, as devices to increase the productivity of a culture for secondary metabolite formation or production of organized tissues (Shuler, 1988). Bioreactors have the potential to resolve the manual handling of the various stages of micropagation due to their automation as well as to decrease production cost significantly (Debnath, 2007). There are different bioreactors or modifications that exist: some examples are the Recipient of Automated Temporary Immersion System (RITA®), pre-sterilised plastic airlift bioreactors (LifeReactor™, Osmotek, Rehovet, Israel), and temporary root zone immersion (TRI). All temporary immersion systems consist of two chambers, the lower chamber normally being used as the reservoir for the nutrient solution, and the upper

one for culturing the explants. A narrow air distribution chamber is located between these two chambers. There are two port channels for air inlet and air outlet on the lid of the vessel. The air inlet tube is connected to an air pump via a filter disc to prevent microbes entering the culture vessel while the air outlet, also connected via a filter, helps to eliminate toxic airborne compounds from the vessel. To supply the explants with the liquid medium the pump is switched on which raises the pressure in the headspace of the nutrient reservoir and forces the nutrient solution from the reservoir into the culture chamber. The pump is connected to an electric timer that allows the system to have a flush and rest period that is fully automatic. When the flushing period is over, the excess nutrient solution flows back into the reservoir under gravity. There have been several studies that prove the potential of the bioreactor system for large-scale micropropagation (Levin and Vasil. 1989; Afreen *et al*., 2002; Ziv, 2005; Debnath, 2007).

Although tissue culture has many advantages, one disadvantage is somaclonal variation, i.e. the genetic variation produced by tissue culture techniques (Scowcroft and Larkin, 1988). The genotype of the explant, culture regimes and plant growth regulators can influence the cytological status of cultured cells. Therefore, it is common practice to assess the trueness to type of *in vitro* plants after regeneration. However, in some systems one of the benefits of somaclonal variation is the creation of additional genetic variability in co-adapted, agronomically useful cultivars, without the need for hybridization (Scowcroft and Larkin, 1988). The risks of somaclonal variation of different tissue culture types are different. A comparison amongst culture types clearly indicates that direct organogenesis, such as apical and axillary shoot culture, is the safest method because explants maintain their developmental integrity in culture. Conservation studies normally utilize explants that maintain the genetic integrity of the species in culture.

There are several factors implicated in the induction of organogenesis and somatic embryogenesis. These are the origin of the explant, media composition, and the response of the explant to exogenously applied plant growth regulators (Terzi and Loschiavo, 1990).

1.2.1.1 Explant choice and decontamination

An explant is referred to as the initial piece of tissue introduced *in vitro*. Given the totipotent characteristic or plasticity of plant cells, virtually any part of the plant can be cultured *in vitro* (George, 1993). However, in most instances the availability of plant material as well as the objective of the study determines the choice of explant. The physiological age of the explant is one of the factors that exercises an influence on organ formation (Narayanaswamy, 1977). Juvenile tissues of trees are most commonly used for *in vitro* propagation since mature tissues are not as receptive to *in vitro* manipulation (Paranjothy *et al*., 1990; Zoglauer *et al*., 2003). Seasonal variations also influence the regenerative capacity of the explant.

One of the main hurdles to overcome in tissue culture is the elimination of fungi and bacteria normally associated with explants from *ex vivo* origin (Debergh *et al*., 1990). The presence of microorganisms associated with the explant could be both external (surface infection) and internal (systemic infection). There are several sterilants that are most commonly used in tissue culture practice (Table 1.1).

Table 1.1: Disinfectants commonly used for decontaminating plant material (1-8 from Razdan, 2002).

No.	Disinfectant	Concentration	Duration of treatment (min)
1	Benzalkonium chloride	0.01-0.1%	5-20
2	Bromine water	1-2%	2-10
3	Calcium hypochlorite	1-10%	5-30
4	Ethyl alcohol	75-95%	Several seconds to several minutes
5	Hydrogen peroxide	3-12%	5-15
6	Mercuric chloride	0.1-1.0%	2-10
7	Silver nitrate	1%	5-30
8	Sodium hypochlorite	0.5-5%	5-30
9	*Triazole	0.5-1%	Several minutes to hours
10	*Propamocarb-HCl	2.5-5%	Several minutes to hours

11	*Fludioxonil	0.33-0.5%	Several minutes to hours
12	*Azoxystrobin	0.8-1%	Several minutes to hours

* Fungicides currently used in the Plant Germplasm Conservation Laboratory at the University of KwaZulu-Natal, Howard College, Durban.

In some cases, the sterilants listed in Table 1.1 (No's 1-8) are not effective in eliminating microbial contamination from explants. This may be because the sterilants are able to remove contaminants from only the surface of the explant; but many explants have systemic infections. In such cases, the use of various systemic fungicides (Table 1.1, no's 9-12) is necessary or antibiotics (for bacterial elimination). Explants may be pre-treated with an antibiotic (40-50 mg l^{-1}) for 30-60 mins or antibiotics can be added into the culture medium (Razdan, 2003). There are various groups of fungicides; systemic and non-systemic that can be used in isolation or combinations (fungicidal cocktails) to eliminate severe fungal infections.

Tissue culture procedures are usually carried out in a laminar airflow cabinet. The large numbers of fungal spores in our environment contributes to the contamination problems encountered *in vitro*. Each human hair can hold 20 to 30 spores and a leaf of paper up to 1000 spores, while the collection of garbage cans adds billions of spores to the immediate vicinity (Debergh *et al*., 1990). The laminar airflow cabinet has a small motor that blows air into the unit first through a coarse filter, where large dust particles are trapped; the air subsequently passes through a 0.3 µm high-efficiency particulate air (HEPA) filter (Razdan, 2003). The air is directed either downward (vertical flow unit) or outward (horizontal flow unit) maintaining an ultraclean airflow, free from fungal and bacterial contaminants (Razdan, 2003).

Autoclaving is the most frequently used method for decontaminating culture media and glass culture vessels, and is based on the mechanism of sterilising with water vapour under high pressure. The material in the autoclave is heated to 121˚C at a pressure of 1 Kpa for 15-20 min, which is sufficient to kill nearly all microbes present (Razdan, 2003). For culture media, distilled water, micro-and macronutrients, and other stable mixtures are autoclaved, whereas the solutions that contain thermolabile compounds are

filter-sterilised. A filter with a pore size of not more than 0.22 µm is most commonly used in filter sterilisation. During the course of aseptic procedures, instruments are usually sterilised to minimise the spread of any contamination that might exist. Instruments are either flame-sterilised or, alternatively, can be dry-sterilised. Flame sterilisation requires the dipping of the end of the instrument into 95-100% Ethanol (EtOH) and flaming using a Bunsen/spirit burner followed by cooling. For dry sterilisation, instruments can be placed in a glass bead steriliser or “steri pot” filled with glass beads that is set to reach a temperature of approximately 250°C. This procedure is safer since the hazards of working with flammable chemicals are removed.

Another prevalent problem when initiating cultures is the exudation of phenolics from the cultured explant (George, 1996). Phenolics exudation is diagnosed by browning of the culture medium in the vicinity of the explant, and ultimately results in tissue death, or poor performance of the explants *in vitro*. In certain species, the decontamination protocol could be responsible for the blackening of the explant or, more commonly, phenolics production is a reaction to wounding (George, 1996). Minimising damage to the explants during excision from the parent plant can reduce the production of phenolics. Other mechanisms that can be utilized to reduce phenolics exudation include treating the explant with an anti-oxidant solution, trimming of darkened edges, the use of liquid media, culturing on a porous substrate, frequent subculturing as well as the inclusion of activated charcoal into the culture medium (George, 1996)**.** The incorporation of anti-oxidants, usually ascorbic acid, in the culture medium or anti-oxidant soaks (ascorbic acid and citric acid) prior to sterilization procedures has been beneficial in reducing the browning of the culture medium (Flynn *et al*., 1990). Anti-oxidants act as electron donors, which inhibit the oxidation of labile substrates with a high stoichiometric efficiency (George, 1996).

1.2.1.2 Medium composition

The successful establishment of any tissue culture protocol depends largely on the composition of the culture medium (Narayanaswamy, 1977; Razdan, 2003). The objective of any study, whether it is to achieve an optimum growth rate or induce either organogenesis or embryogenesis, determines the formulation of the growth medium

since each tissue type requires a different formulation (Narayanaswamy, 1977). Several workers have developed media formulations to suit particular requirements of a cultured tissue. White's medium is one of the earliest tissue culture medium formulations used for root cultures. For the induction of organogenesis and plantlet regeneration in cultured tissues, MS (Murashige and Skoog, 1962) and LS (Linsmaier and Skoog, 1965) contain the desired salt composition and are widely used. The MS and LS media formulations different only in their content of organic nutrients. The use of WPM (Lloyd and McCown, 1981) has been considered appropriate for shoot tip and axillary bud cultures (Wilhelm, 2003).

A standard basal medium consists of a balanced mixture of macronutrient and micronutrient elements (salts of chlorides, nitrates, sulphates, phosphates, and iodides of Ca, Mg, K, Na, Fe, Mn, Zn, and boron), vitamins (thiamine (B_1), nicotinic acid (B_3), pyridoxine (B_6), calcium pentothenate (B_5) and myo-inositol), and a carbon source, organic growth factors (aminoacids, urea and peptones), a source of reduced nitrogen supply, gelling agents and plant growth regulators (Narayanaswamy, 1977; Razdan, 2003). Vitamins are endogenously synthesized and are implicated in various metabolic processes but some essential vitamins are synthesized only in suboptimal quantities thus necessitating the inclusion of exogenously applied vitamins. Thiamine is considered the most basic vitamin required by all cells and tissues (Razdan, 2003).

The most common carbon source included in the growth medium is sucrose. Glucose is equivalent to sucrose in terms of supporting good growth but fructose is less efficient. Other carbohydrates, such as lactose, galactose, rafinose, maltose, meliobiose, celliobiose and trehalose can also be incorporated to the medium but generally produce inferior results (Razdan, 2003). The growth medium is usually solidified with gelling agents to prepare semisolid and solid media. The most common gelling agent used is agar, a polysaccharide, based on the advantages that agar does not react with other media constituents, does not get digested by plant enzymes and remains stable at all incubation temperatures. Other compounds used include methacel, alginate, phytagel and Gelrite®. Depending on the tissue and species certain gelling agents can support better growth than others. Other additives that can be beneficial in tissue culture but are

not essential include deproteinized coconut milk, tomato juice, yeast extract, malt extract and protein hydrolysate, all which bring about mitosis in quiescent cells (Narayanaswamy, 1977).

Growth regulators can be classified into four broad classes, auxins, cytokinins, gibberellins and abscisic acid. These regulators control the growth, differentiation and organogenesis of tissue when added to the growth medium singularly or in combination with the other classes (Razdan, 2003). Auxins are naturally associated with elongation of the stem, internodes, tropism, apical dominance, abscission and rooting. There are various auxins that can be incorporated to the growth medium: indole-3-acetic acid (IAA), 1-naphthalene-acetic acid (NAA), indole-3-butyric acid (IBA), 2,4-dichlorophenoxyacetic acid (2,4-D), naphthoxyacetic acid (NOA), 4-chlorophenoxyacetic acid (4-CPA), 2,4,5-trichlorophenoxyacetic acid (2,4,5-T), 2-methyl 4-chlorophenoxyacetic acid (MCPA), 4-amino-3,5,6-trichloropicolinic acid (picloram), and 3,6-dichloro-2methoxybenzoic acid (dicamba). The various auxins different in their physiological activity and their extent of mobility through tissue or attachment to cells, the most commonly used being 2,4-D employed for the induction of callus (Razdan, 2003).

Cytokinins are adenine derivatives which function to bring about cell division, modification of apical dominance and shoot differentiation of cultured tissue. Frequently used cytokinins are 6-benzylaminopurine (BAP), 2-isopentenylamino purine (2-iP), ^{2}N-(2-furfurylamino)1-H-purine-6-amine (kinetin), 6-(4-hydroxy-3-methyltrans-2-butanylamino) purine (zeatin) and thidiazuron (TDZ). Synthetic cytokinins are BAP and kinetin while zeatin and 2-iP are naturally-occurring cytokinins. The ratio of auxin to cytokinin plays a vital role in morphogenesis (Narayanaswamy, 1977; Merkle *et al.*, 1990; Tran Thanh Van and Trinh, 1990; Razdan, 2003). For the propagation procedures of embryogenesis, callus initiation and root initiation the requisite ratio of auxins to cytokinin is high, while the reverse leads to axillary and shoot proliferation (Zhang and Lemaux, 2004). The most common gibberellin used is GA_3 which acts to promote the growth of cell cultures at low density, enhances callus growth and induces dwarf or stunted plantlets to elongate. Abscisic acid (ABA) either stimulates or inhibits callus

growth depending on the species, when added to the culture medium. ABA has been used in somatic embryo studies to promote maturation of embryos (Von Aderkas *et al*., 1995; Yildirim *et al*., 2006; Kim and Moon, 2007).

Temperature, photoperiod, light intensity and pH are other factors that influence organogenesis and embryogenesis. Incubation temperatures of cultures usually depends on the provenance of the species. The photoperiod used for most cultures is 16 h light and 8 h dark; however, some investigations rely on the incubation of cultures in continuous darkness for certain periods which may be very prolonged (Reuveni and Evenor, 2007). Incubation of cultures in the dark can aid in rooting as well as shoot regeneration (Mohamed *et al*., 2006). Exposure of cultures to light of different intensities and wavelengths can control the nature of differentiation in a tissue and should also be taken into account (Narayanaswamy, 1977; Reuveni and Evenor, 2007). The pH of culture medium is another important factor and should be adjusted to 5.6-6.0 by the addition of 0.1M HCl or NaOH (Narayanaswamy, 1977).

The two *in vitro* methods that can be used for conservation are active cultures and slow growth cultures. The plantlets used for these methods can be generated using the micropropagation procedures discussed above either organogenesis or embryogenesis. These two methods can be viewed as short-to-medium term approaches to germplasm conservation as risks of loss must accumulate with each subculturing operation and during each storage interval (Withers, 1988; Engelmann, 1991, 2000). The advantages of this method, however, are that culture deterioration can be detected visually and therefore loss of viability may be avoided. The space required for the storage of *in vitro* materials is relatively small as compared to that needed for field cultivation. However, disadvantages associated with these methods include high costs, the risk of contamination and genetic or phenotypic modification during subculturing (Mix-Wagner and Schumacher, 2003).

Actively growing cultures depends on the maintenance of plants/shoots *in vitro*. This method requires frequent subculturing to fresh medium to avoid loss of viability. The principle of reduced-growth storage or minimal growth is based on the manipulation of

culture conditions/culture media to allow the cultures to remain viable, but with a growth rate that is very slow (Dodds, 1991). Reduced growth can be achieved by the manipulation of culture media; the reduction of incubation temperatures; the combination of both temperature reduction and culture medium manipulation; and the modification of the gaseous environment (Ng and Ng, 1991). Manipulations of culture media are usually associated with raising the osmolarity of the medium, for instance the inclusion of mannitol to the medium and/or including growth retardants, e.g. ABA (Benson and Withers, 1988). The most frequent method employed is the reduction of temperature, often in combination with a high osmolarity medium (Staritsky, 1997).

1.2.2 Base collection

Maintenance of base collections by means of the *ex situ* conservation method involves a process called cryopreservation. Cryopreservation (preservation in the frozen state at temperatures well below zero) is based on the reduction and subsequent arrest of metabolic functions of biological material by the imposition of ultra-low temperature (Grout, 1990; Razdan and Cocking, 1997). At the temperature of liquid nitrogen (-196°C) all the metabolic activities of cells are expected to be halted and the specimen should be preserved for indefinite periods (Kartha, 1987). Although affording long-term preservation, Walters *et al.* (2005) have reported that viability of cryopreserved explants does slowly decline, and Benson and Bremner (2004) detail phenomena that can occur, even at liquid nitrogen temperature.

Nevertheless, cryopreservation represents the only feasible option for long-term conservation of the germplasm of species producing non-orthodox seeds, vegetatively propagated species, and of biotechnology products (Kartha, 1987; Withers, 1988; Dumet *et al.,* 1997; Engelmann, 1997; Engelmann, 2000)**.**

Successful *in vitro* conservation of explants via cryopreservation necessitates the optimisation of various parameters before storage in liquid nitrogen is possible. Each stage in the cryopreservation procedure involves potential sources of stress and injury (Withers, 1988; Berjak *et al.*, 1999). Over the years, considerable efforts have been made to preserve species that cannot be stored using conventional storage methods

(Engelmann, 1997). However to date, there has not been the development of one single method or strategy that can be used universally for successful cryopreservation (Jun-Yan *et al*., 2000). This is due to the multitude of diversity across species and the varied responses to different treatments and manipulations involved in cryopreservation. Protocols have to be developed on a species-specific basis, taking into account the material that will be frozen, the age of the material, and the natural growing environment of the plant species (Engelmann, 1997). The preservation of explants from temperate species has met with much more success compared with that of tropical species (Berjak and Pammenter, 2007). This can be attributed to tissues of tropical species being less cold and desiccation tolerant than those of temperate species (Fang *et al*., 2003).

The main factors for developing a cryopreservation protocol are firstly choosing the right explant and establishing an *in vitro* regeneration protocol for the explant of choice, and thereafter, optimizing a pre-treatment, dehydration and cooling strategy.

1.2.2.1 Choice of Explant

Explants chosen for cryopreservation should be in an excellent physiological state suitable for the acquisition of osmotolerance and the ability for vigorous growth recovery (Sakai, 2000). The ideal explant for cryostorage would be an intact seed, as is used for orthodox types (e.g. Walters *et al*., 2005) and has been used successfully for non-orthodox seeds of for *Azadirachta indica* (neem) (Chaudhury and Chandel, 1991; Berjak and Dumet, 1996) and *Warburgia salutaris* (Kioko *et al*., 2003). However, this is exceptional, as difficulties generally arise with non-orthodox seeds because of their size, the high water contents at shedding and sensitivity to desiccation (Dumet *et al*., 1997). Many recalcitrant species, especially large-seeded tropical species with large embryos, present particular problems for the application of any drying and freezing protocols (Pence, 1995b).

In cases were intact seeds are not suitable for cryopreservation embryonic axes may be used. In most cases, the embryonic axis only constitutes a small proportion of the total seed mass, so, after excision, provides a sufficiently small explant (Berjak *et al*., 1998).

Successful protocols have been established for excised embryonic axes of *Camellia sinensis* (Wesley-Smith *et al*., 1992), longan (Fu *et al*., 1993), *Quercus robur* (Berjak *et al*., 1999), chayote (Abdelnour-Esquivel and Engelmann, 2002), *Castanea sativa* (Corredoira *et al*., 2004) and *Poncirus trifoliata* (Wesley-Smith *et al*., 2004). The embryo is excised from the seed and, after cryopreservation, must be established *in vitro*.

Normally, *in vitro* germination of mature zygotic embryos is performed on simple nutrient media. However, in some instances, the use of growth regulators is required (Smith and Drew, 1990; Dumet *et al*., 1997). It is imperative to develop appropriate tissue culture techniques for reliable regeneration of plants (Kartha, 1987) and this needs to be done before any manipulation for cryopreservation is attempted. Cryopreservation of many recalcitrant-seeded species via zygotic embryos has been precluded by the lack of shoot formation post freezing e.g. (Kioko *et al*., 1998), and this appears to be particularly true for tropical/sub-tropical species (e.g. Goveia *et al*., 2004). According to Engelmann (1997) zygotic embryos appear to be generally more sensitive to freezing at the shoot pole as compared to the root pole. Another factor precluding the use of zygotic embryos of some species may be their large size and/or undifferentiated hypertrophied nature for example, *Theobroma cacao* and *Barringtonia racemosa*.

In addition, when using explants that are desiccation sensitive it has been shown that material dried rapidly (tens of minutes to hours) can survive to lower water contents before viability is lost than material that is dried slowly (over a period of days) (Pammenter *et al*., 1991, 1998). Material dried rapidly passes through intermediate water contents that can cause damage to the explant so fast that there is no time for the accumulation of these damaging processes and tissue viability is maintained (Pammenter *et al*., 1998; Pammenter and Berjak, 1999). Therefore, smaller thinner explants are favoured since these explants have less tissue to be dehydrated and can be desiccated rapidly as compared with larger thicker explants that will require longer dehydration periods to achieve suitable water contents for cooling (Pammenter *et al*., 2002). It is necessary to reduce the thermal mass (a combination of explant size and

water content) for effective, non-injurious cooling in liquid nitrogen (Pammenter *et al*., 1998).

Other types of explants need to be generated in instances where zygotic embryos are not amenable to the procedures involved in cryopreservation – for whatever reason; these can fall into two categories a) organised cultures (somatic embryos, shoot tips, axillary buds, nodal segments and anther/ovule cultures) or b) unorganised cultures (callus, cell suspensions and protoplasts) (Engelmann, 1997). When the choice of explant is an organised structure, it is important to choose young meristematic tissues, the cells of which have compact non-vacuolated cytoplasm and thus reducing the amount of water available for ice formation (Benson and Withers, 1988). Recently, there have been many successful cryopreservation attempts using these types of explants (Table 1.2). The size of the explant to be cryopreserved plays a crucial role in achieving successful cryopreservation, as explained above. This was demonstrated for shoot apices by Baek *et al*. (2003) of garlic where smaller apices (1.5 or 3.0 mm in diameter) displayed higher regeneration frequencies than larger ones (4.5 mm in diameter).

Table 1.2: Some cryopreserved explants and the cryopreservation techniques used.

Species	Explant frozen	Technique	Reference
Aesculus hippocastanum (Horse chestnut)	Embryonic axes	Rapid freezing	Wesley-Smith *et al.* (2001)
Arachis hypogaea, A. retusa and A. burchellii	Shoot tips	Vitrification	Gagliardi *et al.* (2003)
Asparagus	Shoot tips	Freeze droplet method	Mix-Wagner *et al.* (2000)
Carica papaya (Papaya)	Shoot tips	Vitrification	Wang *et al.* (2005)
Castanea sativa (Chestnut)	Somatic embryos Shoot tips	Vitrification	Corredoira *et al.* (2004) Vidal *et al.* (2005)
Colocasia esculenta (Tropical taro)	Shoot tips	Vitrification	Sant *et al.* (2006)
Dioscorea spp. (Yams)	Apical buds	Vitrification-droplet method	Leunufna and Keller (2005)
Eucalyptus grandis	Axillary buds	Encapsulation	Blakesley and Kiernan (2001)
Fragaria cultivars (Strawberry)	Apices	Encapsulation-dehydration	Clavero-Ramírez *et al.* (2005)
Fraxinus excelsior (Ash)	Shoot tips	Vitrification	Schoenweiss *et al.* (2005)
Garlic	Shoot tips	Vitrification	Baek *et al.* (2003) Kim *et al.* (2004) Volk *et al.* (2004) Keller (2005)
Gentiana scabra, G. triflora and *G. pneumonanthe*	Shoot tips	Vitrification Encapsulation-Vitrification	Tanaka *et al.* (2004)

Manihot esculenta Crantz (Cassava)	Shoot tips	Vitrification	Charoensub *et al*. (2004)
Musa spp. Banana	Meristems	Sucrose pre-culture, Simple freezing Rapid cooling	 Ramon *et al*. (2002) Panis *et al*. (2002)
Picrorhiza kurroa	Shoot tips	Vitrification	Sharma and Sharma (2003)
Poncirus trifoliata and *Citrus sinensis*	Shoot tips	Vitrification Encapsulation-dehydration	Wang *et al*. (2003)
Solanum tuberosum (Potato)	Shoot tips Axillary buds Shoot tips	Sucrose pre-culture, cold acclimation, Vitrification. Cold acclimation, Vitrification with Supercool X1000	Halmagyi *et al*. (2005) Zhao *et al*. (2005)
Sechium edule (Chayote)	Shoot tips	Vitrification	Abdelnour-Esquivel and Engelmann (2002)
Solemostemon rotundifolius (Innala)	Lateral buds	Vitrification	Niino *et al*. (2000)
Syzygium francissi	Shoot tips	Encapsulation-Vitrification	Shatnawi *et al*. (2004)
Vitis vinifera (Grape)	Axillary buds	Encapsulation dehydration	Zhao *et al*. (2001)
Zizania texana	Somatic embryo	Cryoprotectants	Walters *et al*. (2002)

Once the choice of explant is made and a working protocol is developed, the next phase of the investigation can be optimised. As mentioned above, during cooling free water within the tissue forms ice crystals, which have the potential to cause lethal mechanical damage to cell membranes and organelles (Dumet *et al*., 1997; Volk and Walters, 2006). In an attempt to reduce the amount of freezable water (Vertucci and Leopold, 1987) from the tissue, the explant needs to be pre-treated, which should lower the water content to some extent, and subsequently desiccated further (Dumet *et al*., 1997).

1.2.2.2 Pre-growth

Pre-growth provides the opportunity for selecting or inducing the most freeze-tolerant growth phase (Withers, 1988). Pre-growth, which involves placing explants on medium with additives such as particular amino acids, mannitol, sorbitol, sucrose, abscisic acid or dimethyl sulphoxide (DMSO), can enhance freeze-tolerance (Benson and Withers, 1988; Withers, 1988). Cellular activity during pre-growth is associated with changes in cell size, lessening the degree of vacuolation and cell wall flexibility, which are involved in facilitating freeze tolerance (Withers, 1988). Incorporation of high sucrose or mannitol concentrations in the culture medium has been used to improve cryotolerance of e.g. somatic embryos, meristems and nodal segments (Dumet *et al*., 1993; Niino *et al*., 2000; Panis *et al*., 2002; Quain *et al*., 2007). The optimal sucrose concentration in the pre-culture medium is usually between 0.3 - 0.5 M (Baek *et al*., 2003).

Cold acclimation of *in vitro* material prior to freezing has also shown indications of increasing post freezing survival (Kamata and Uemura, 2004; Keller, 2005; Halmagyi *et al*., 2005; Schoenwiss *et al*., 2005; Sharma and Sharma; 2003; Zhao *et al*., 2005). A cold pre-culture of two months at 2˚C for garlic shoot cultures resulted in high survival rates of 70% using the vitrification technique (Keller, 2005). A study on different wheat genotypes showed an increase in the accumulation of sugars, amino acids and glycinebetaine in leaf tissues during a cold acclimation pre-culture of 4 weeks in a growth chamber at 3˚C (Kamata and Uemura, 2004). Those authors documented a good relationship between the degree of freezing tolerance and osmotic concentration occurring during cold acclimation in the different wheat cultivars.

1.2.2.3 Cryoprotection

While much of the work to date on zygotic axes has been done in the absence of cryoprotectants (Fu *et al*., 1993; Dumet and Berjak, 2002), to minimise the damage caused by dehydration, explants may be treated with protectants. Cryoprotectants are used primarily to reduce freezing injury of hydrated tissues. Cryoprotectants can be categorized as penetrating and non-penetrating. Penetrating cryoprotectants have the beneficial effect of maintaining cellular solutions in a liquid state at low temperatures preventing an excessive concentration of toxic solutes in the non-frozen cellular structures (Benson and Withers, 1988). Non-penetrating cryoprotectants are associated with promoting dehydration and reducing the amount of intra-cellular water available for ice formation (Benson and Withers, 1988).The protectants commonly used, either singly or in combination, include glycerol, dimethylsulphoxide (DMSO), polyethylene glycol (PEG), sugars and sugar alcohols (Kartha and Engelmann, 1994). Cryoprotecting explants prior to dehydration or freezing has the beneficial effects of stabilizing membranes and macromolecules through various mechanisms, the most significant of which may be free radical scavenging (Benson and Withers, 1987). However, cryoprotectant toxicity is a well-recognized problem that is difficult to resolve totally. Therefore, over-administration should be avoided and cryoprotectant mixtures used to give an overall protective effect without additive toxic effects (Withers, 1988).

1.2.2.4 Desiccation

The optimal water content for cryopreservation is generally between 0.11 g g^{-1} and 0.19 g g^{-1} (dry-weight basis) (Engelmann, 1992). However, depending on the species and the type of explant, optimal water contents vary (Dumet *et al*., 1997). The drying of explants can be achieved by using various methods, achieving different drying rates. These methods include: (a) a sterile stream of air in a laminar flow cabinet; (b) activated silica gel in airtight containers, (c) flash-drying (Berjak *et al*., 1990) (d) under vacuum (Fu *et al*., 1990) (e) the use of saturated salt solutions. The rate of drying plays a crucial part in the amount of desiccation tolerated. Rapid drying (e.g. by flash-drying) increases the amount of dehydration tolerated before viability is lost, simply by swiftly curtailing damaging metabolism-linked damage (Pammenter *et al*., 1998, Walters *et al*., 2001)

1.2.2.5 Cooling

Cooling may be carried out slowly or rapidly, and the rate of cooling has a major influence on the survival of cryopreserved plant tissue (Withers, 1988). Cooling can cause damage resulting from both dehydration (via extracellular ice nucleation) and ice formation. While slow cooling of hydrated tissues carries the attendant risk of lethal intracellular ice crystallization, very rapid cooling rates are generally held to minimise this, as the tissues pass extremely rapidly through the temperature range at which such crystallization occurs (Wesley-Smith, 2004). Slow cooling may be attained by using a programmable freezing apparatus, achieving a cooling rate of 0.5 to 1.0˚C/min (Kartha and Engelmann, 1994). Alternatively, specimens can be pre-cooled at a temperature of -18˚C in a deep freezer prior to immersion in liquid nitrogen (step-wise freezing). Slow cooling has been suggested to be associated with damage by causing membrane lesions (Withers, 1988) and disruption of the symplasm by breakage of plasmosdesmatal connections between adjacent cells (Benson and Withers, 1987). A study by Wesley-Smith (2001) revealed however, that damage revealed microscopically could actually be an artifact of fixation of the frozen material.

A more rapid cooling rate can be achieved by enclosing specimens in either aluminium foil or wire mesh pockets, which are then rapidly plunged into liquid nitrogen. Wesley-Smith *et al*. (1992) have attained extremely rapid cooling rates - around 500˚C s^{-1}- for hydrated axes of *Camellia sinensis* by using a spring-loaded device that propelled the axes into sub-cooled nitrogen (-210˚C). Cooling rates attained depend on the volume of the explant, its water content (the thermal mass) and the mode of explant introduction into the cryogen (Wesley-Smith *et al*., 1992). It also appears that the composition of explant tissues has a bearing on the most favourable cooling rate for that explant-type (Wesley-Smith, 2004). Although vegetative explants are usually cooled relatively slowly in cryopreservation procedures, rapid cooling has been successfully used for banana meristems (Panis *et al*., 2002).

1.2.2.6 Thawing and recovery of frozen material

Thawing of frozen tissues is generally a very rapid procedure, in order to avoid ice recrystallization damage which will occur during the passage of the tissues through the

same temperature range facilitating crystallization during cooling (Withers, 1988). Thawing of frozen tissue is also associated with deplasmolysis injury (Withers, 1988).

Plant material retrieved from cryopreservation may require some additives to the recovery medium for better survival when recultured (Withers, 1980; Krishnapillay, 2000). Frozen-thawed cells differ from unfrozen original control cells, physiologically (Cella *et al*., 1982) and structurally (Withers and Street, 1976). Therefore, culture conditions most suitable for frozen-thawed cells are likely to differ somewhat from those of unfrozen original control cells (Watanabe, 2000). It appears that the use of growth regulators in the recovery medium is beneficial for organised structures, which often fail to recover in the absence of hormones, and the use of auxins and cytokinins has been reported to improve the survival of post-thaw explants (Benson and Withers, 1987; Turner *et al*., 2001; Wang *et al*., 2003). However, inclusion of growth regulators should be used with caution since they can often promote destabilizing phenomena such as callusing and adventitious shooting (Benson and Withers, 1988).

1.2.2.7 Cryopreservation techniques

Cryopreservation techniques can be classified into two: classical techniques, and new vitrification-based techniques. Classical techniques involve freeze-induced dehydration while vitrification relies on the transition of water directly from the liquid phase into an amorphous or glassy phase, avoiding the formation of ice crystals (Fahy *et al.,* 1984; Sakai, 1990).

Classical cryopreservation techniques involve slow cooling down to a specific prefreezing temperature, followed by rapid immersion into liquid nitrogen. This method encourages extracellular ice formation, whilst increasing intracellular solute concentrations. The amount of water that leaves the cell before the intracellular contents solidify is dependent on the rate of cooling as well as the prefreezing temperature (Engelmann, 1997). Ideally, most or all intracellular freezable water is removed to avoid the formation of intracellular ice formation before subsequent immersion into liquid nitrogen, although biochemical damage could be incurred with too intense freeze-induced dehydration (Mazur, 1984). However, when optimised, this method has been

employed successfully using unorganised culture systems, calli and cell suspensions (Kartha and Engelmann, 1994).

Vitrification-based procedures rely on cell dehydration prior to freezing by exposure to a concentrated cryoprotective solution (Fahy *et al*., 1984). According to Sakai (1995, 1997) vitrification may be the only freeze-avoidance mechanism that allows hydrated cells and organs to survive at the temperature of liquid nitrogen, although Wesley-Smith *et al*. (1992, 1998, 2001) have achieved this by very rapid cooling in the absence of cryoprotectants. Since the induction of the various vitrification-based freezing techniques, explants of more species than before, have been successfully cryopreserved (Engelmann, 2000). By precluding ice formation, vitrification-based procedures simplify the cryogenic procedure and eliminate concerns about the potential dangers imposed by ice formation. High survival and post-freezing regrowth have been reported, and vitrification procedures are held to have greater potential for varied applicability, requiring minute modifications for diverse cell types (Engelmann, 1997; Sakai, 1997; Volk and Walters, 2006). There are currently seven different vitrification-based procedures: (1) encapsulation-dehydration; (2) vitrification; (3) encapsulation-vitrification; (4) desiccation; (5) pre-growth; (6) pre-growth and desiccation, and (7) droplet freezing (Engelmann, 1998).

2. Species of interest in this investigation

The present study was focused on the germplasm of two species, *Theobroma cacao* L. and *Barringtonia racemosa* (L.) Roxb, both of which produce seeds with axes/embryos that are not amenable to cryopreservation.

2.1 *Theobroma cacao*

Cocoa is produced from the seeds of *Theobroma cacao*, a tree that belongs to the family Sterculiaceae (George, 1993). It is an understory tree of tropical moist forests and can reach a height of 12 m. The canopy is made up of branches bearing oblong leathery leaves that can extend to 30 cm in length (Verzeletti, unknown). Cocoa trees require constant warmth and rainfall to survive and are maintained in a temperature regime between 18˚C and 32˚C. They begin to produce their first fruit, the so-called ‘pods’

(Figure 1.1), after three to five years (Verzeletti, unknown). The flowers are pink to whitish and grow along the trunk and main branches. There are three varieties or groups of cocoa, i.e. Criollos, Forastero and Trinitario. Trinitario is a combination of Criollos and Forastero and it is probably the most widely cultivated variety today (Pence, 1989).

The cotyledons of cocoa seeds contain the precursors of chocolate, but must undergo fermentation, drying and roasting to reach full cocoa flavour (Pence, 1995a). The seed also contains approximately 50% fat, which, when expressed, is known as cocoa butter (Pence, 1995a). These properties of cocoa provide the basis of multibillion US Dollar industries, which have significant economical influences on countries that supply cocoa to the rest of the world. The major cocoa producers are Cộte d'Ivoire, Brazil, Ghana and Malaysia (Pence, 1989). The demand for cocoa is growing and there is concern as to whether the world's supply of a product that comes from a strictly tropical, rainforest tree will continue to meet this demand.

Figure 1.1: Cocoa fruit and seeds. Scale bar = 15 mm

Over the past two decades, several serious fungal diseases (which are mainly manifested on/in the cocoa pods) have increasingly posed a serious threat to the supply of cocoa beans. Of these diseases, black pod is the most widespread, causing the destruction of the cocoa crop worldwide. It is caused by several species of the fungus *Phytophthora* (Bowers *et al*., 2001). West and Central Africa are the areas most affected by black pod and it is these regions that contribute 60-70% of the world's production of cocoa beans (Bowers *et al*., 2001). Other diseases include witches' broom caused by *Crinipellis perniciosa*, a fungus, that debilitates cocoa trees by diverting energy to the numerous 'brooms' that do not form flowers, thus reducing yield potential; frosty pod rot caused by *Moniliophthora roreri* that destroys the cocoa beans inside the pods (Bowers *et al*., 2001); as well as vascular streak dieback and swollen shoot virus (Bowers *et al*., 2001). Altogether, 81 000 tons of cocoa are estimated to be lost annually resulting in an economic loss of 76.1 million US Dollars (Bowers *et al*., 2001).

2.1.1 *In vitro* propagation studies on cocoa

There have been many contributions to the micropropagation of cocoa germplasm. Many researchers have carried out experiments on somatic embryo induction using different explants, various growth regulators and multi-step procedures (Table 1.3). The multistep procedure incorporated in recent studies involves the induction of callus on one type of basal medium and the subsequent transfer of the callus onto another basal medium for somatic embryogenesis and thereafter transfer onto the initial basal medium devoid of growth regulators for embryo maturation (Li *et al*., 1998; Guiltinan *et al*., 2000; Tan and Furtek, 2003).

The basal salts play an important role in the induction of somatic embryogenesis (discussed in 1.2.1.2). The frequently used Murashige and Skoog (1962) medium was found to be inadequate in later studies of somatic embryo induction for cocoa (Li *et al*., 1998; Tan and Furtek, 2003). DKW salt formulation (Driver and Kuniyuki, 1984) which has a higher concentration of calcium, sulphur and magnesium compared with MS basal medium, proved more suitable for the induction of embryos (Li *et al*., 1998; Tan and Furtek, 2003). Even though there is a high multiplication rate, the applicability of embryogenesis is limited by the vague nature of embryogenic cultures, the long period

from culture initiation to embryo production (6-8 months), and the relatively high cost per plant produced (Traore *et al*., 2003).

Even though there has been progress over the years, to date the protocols developed have resulted in very low frequencies of somatic embryos when the method for a particular genotype was applied to other genotypes (Tan and Furtek, 2003). Li *et al.* (1998) observed a broad range of responses by genotypes in both the regularity of embryogenesis, which ranged between 1-100%, and the average number of somatic embryos formed from each responsive explant, which ranged from 2-46.

Table 1.3: Summary of research on the initiation of somatic embryos of *Theobroma cacao* (Pence, 1995).

References	Explant	Results
Essan (1977)	Immature embryos	Direct embryogenesis
Pence *et al*. (1979, 1980)	Immature embryos	Direct embryogenesis
Kononowicz *et al*. (1984)	Somatic embryos	Embryogenic callus
Kononowicz and Janick (1984)	Somatic embryos	Embryogenic callus
Novak *et al*. (1986)	Immature embryos	Direct and indirect embryogenesis
Litz (1986)	Leaf tissue	Developmentally arrested somatic embryos
Elhag *et al*. (1987, 1988)	Somatic embryos	Embryogenic callus
Adu-Ampomah *et al*. (1988)	Immature embryos	Direct and indirect embryogenesis
Wen (1989)	Immature embryos	Direct embryogenesis
Duhem *et al*. (1989)	Immature embryos	Direct embryogenesis
Dos Santos and Machado (1989)	Immature embryos	Direct embryogenesis
Chatelet and Dufour (1990)	Immature embryos	Direct embryogenesis
Söndahl *et al*. (1989)	Nucellus, immature flower petals	Somatic embryos, multistep procedure
Aguilar *et al*. (1992)	Mature cotyledons	Somatic embryos, two-step procedure
Chatelet *et al*. (1992)	Nucellus, inner integument	Developmentally arrested somatic embryos

Figueira and Janick (1993)	Nucellus	Somatic embryos, multistep procedure
Lopez-Baez *et al*. (1993)	Immature flower	Somatic embryos, multistep procedure
Li *et al*. (1998)	Staminodes	Somatic embryos, multistep procedure
Tan and Furtek (2003)	Unopened flower buds, staminodes	Somatic embryos, multistep procedure

In vitro shoot multiplication of cocoa has been attempted with limited success in the past (Orchard *et al*., 1979; Essan, 1981; Passey and Jones, 1983; Janick and Whipkey, 1985; Flynn *et al*., 1990; Figueira *et al*., 1991; Adu-Amponah *et al*., 1992; Lardet *et al*., 1998). Recently, however, there has been successful micropropagation of cocoa via the proliferation of shoots from nodal and apical meristem explants from somatic embryo-derived plants, with subsequent rooting and acclimatization of plantlets (Traore *et al*., 2003).

2.1.2 Cryopreservation studies on cocoa

Cryopreservation of immature axes was the first report for long-term storage for cocoa germplasm (Pence, 1991a). Two techniques were used; *viz*. slow freezing of hydrated explants and desiccation-fast-freezing in liquid nitrogen. Zygotic embryo survival was assessed by the ability of cryopreserved embryos to form callus and to undergo somatic embryogenesis. The maximum rate of somatic embryogenesis, 29%, occurred from tissues of embryos which were pre-cultured on a 3% sucrose medium, cooled slowly with cryoprotectants before exposure to liquid nitrogen, and recovered on medium containing 3 mg l^{-1} NAA (Pence, 1991a). Further studies were done by Pence (1991b, 1992) to increase the desiccation tolerance of cocoa immature zygotic embryos by treating them with exogenous ABA, fluridone or mefluidide, or culturing embryos on medium with increasing sucrose. These investigations revealed that desiccation tolerance did not develop in cacao embryos, but suggested that ABA and sucrose are both needed for the initiation of events associated with maturation *in vitro*.

Fang *et al*. (2003) attempted cryopreservation of cocoa somatic embryos using the encapsulation dehydration technique with different genotypes. The successful protocol included a 7-day pre-culture with 1 M sucrose and 4 h silica gel exposure. The protocol was developed with one genotype and then it was applied to three other genotypes. The results of those authors showed that recovered cryopreserved SPA-4 early cotyledonary somatic embryos converted to plants at a rate of 33%. The procedure on genotypes EET272, IMC14 and AMAZ12 resulted in recovery frequencies of 25, 40 and 72%, respectively. However, the recovery frequencies did not mean the formation of viable plants but rather explants that showed callusing.

Mature embryonic axes of cocoa were deemed to large to achieve the cooling rates required for successful cryopreservation by Kioko *et al*. (2003). Those authors attempted cryopreservation of the embryonic axes and shoot tips of cocoa. They revealed that cryopreservation of axes and shoot- tips using dehydration-freezing and vitrification-freezing was unsuccessful. Although 65% of explants survived encapsulation in calcium alginate beads containing 1% citric acid and quarter-strength MS however, survival did not lead to plantlet production.

2.2 *Barringtonia racemosa*

Barringtonia racemosa belongs to the family Lecythidaceae, of which Brasil nut (*Bertholletica excelsa*) is also a member. It is extensively spread around East Africa, Southeast Asia and Pacific islands (Anon, 2005). It is a relatively small tree, which has the potential of reaching 20 m or more, with leaves tufted at the ends of stout twigs (Deraniyagala *et al*., 2002). It grows best in swamps with moderate to abundant sunlight (Anon, 2005). The stem can grow to approximately 600 mm in diameter and the flowers are pale pink to cream in colour. *Barringtonia racemosa* produces a single-seeded fruit; the seeds are classified as recalcitrant and are characterised by a hypertrophied axis (Berjak *et al*., 1996). The fruits are green when immature and red to brown when they mature (Figure 1.2). The recalcitrant nature of the seed and the large hypertrophied axes (Figure 1.3) has limited seed-based and conservation studies of this species.

Figure 1.2: Fruits of *Barringtonia racemosa*. Scale bar = 10 mm

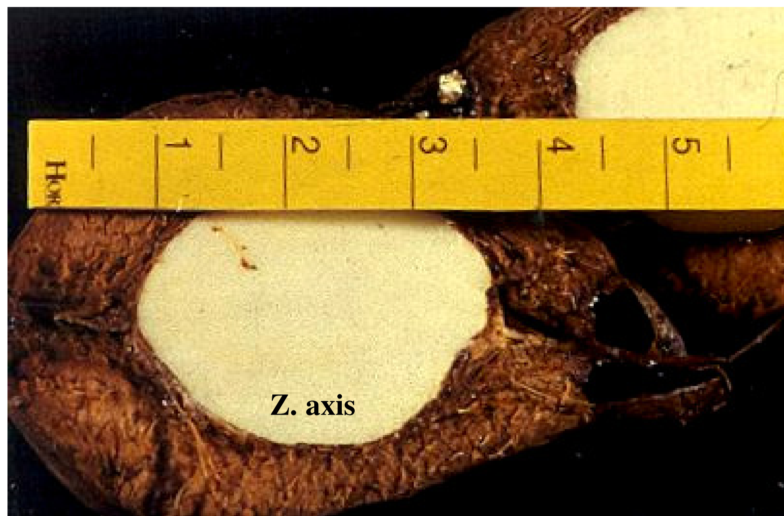


Figure 1.3: Hypertrophied zygotic axis of *B. racemosa*.

Barringtonia racemosa belongs to a genus that can be divided into edible species and non-edible species. The edible species are *B. procera, B. edulis* and *B. novae-hiberniae* indigenous to Solomon Islands, Venuatu and Papua New Guinea and are all commonly referred to as cutnut (Pauku, 2006). Cutnut is a well-known tree in the Melanesian countries. Non-edible species have cultural importance and these include *B. racemosa* and *B. asiatica* (Pauku, 2006). *Barringtonia racemosa* is a good plant for live fences and is used for the treatment of venereal diseases, such as gonorrhea whilst *B. asiatica* is used for fish poisoning and, for treating toothache in the Solomon Islands (Pauku, 2006).

The main form of propagation for these species is via seeds or cuttings (Anon., 2005). The fresh seeds are either directly planted into the field or are first established in a nursery and then transplanted into the field (Pauku, 2006). *Barringtonia procera* has been macropropagated via air layering and stem cuttings successfully in the Solomon Islands. Juvenile stem cuttings set in a non-mist propagator gave 100% rooting in 3 weeks while 100% of air-layers rooted in 4 weeks (Leakey *et al*., 1990).

Barringtonia racemosa is well recognized for its medicinal properties not only in Africa but also in Asia and South America. In Kerala (India), an ethnomedical survey has shown that the seeds of *B. racemosa* have been used to treat cancer-like diseases. Thomas *et al*. (2002) investigated the anti-tumour activity and toxicity in *B. racemosa* seed extracts. The seed extract was found to be deficient of conspicuous acute and short-term toxicity in mice, when administered daily. The concluding suggestion of the study was that the seed extract is non-toxic and can be used for further studies leading to drug development (Thomas *et al*., 2002).

Khan *et al*. (2001) studied the antibacterial properties of the roots to try to justify their use in traditional medicine. The ethanol extract of the roots, its chloroform-soluble fraction and an isolated triterpenoid, nasimalun A, exhibited antibacterial activity against several species of Gram-positive and Gram-negative bacteria. In Sri Lanka, *B. racemosa* is widely used in the form of a decoction in traditional medicine (Deraniyagala *et al*., 2002). The bark and leaves are used for rat and snake bites, for rat

poisoning and on boils (Deraniyagala *et al*., 2002). The seeds, in combination with other ingredients, are used in the preparations for the treatment of itch, piles and typhoid fever. Deraniyagala *et al*. (2002) suggested that this species has compounds with an analgesic effect.

The lack of awareness of the potential economic value of this genus may be a limiting factor in the cultivation as well as the conservation strategies for these species (Pauku, 2006). *Barringtonia racemosa* also belongs to the same family as *Bertholletica excelsa,* commonly known as Brasil nut, which is economically important in certain countries. There are, however, no reports of successful studies aimed at conservation of Brasil nut germplasm. Therefore, if a protocol is established for the long-term storage of *B. racemosa*, this method may be useful in establishing conservation strategies for Brasil nut as well as all the other species of *Barringtonia* mentioned above.

3. Objectives

This study focused on two species, *Theobroma cacao and Barringtonia racemosa*, which share the similarity of being tropical and producing recalcitrant seeds with embryonic axes that are too large and/or undifferentiated to be cryopreserved. Therefore, the aims of the present study were to optimise *in vitro* regeneration protocols for different types of explants (apart from embryonic axes) that have the potential to be cryopreserved and, to develop cryopreservation protocols for these explants.

CHAPTER 2: STUDIES ON *Theobroma cacao* L.

Theobroma cacao, a tropical rain forest tree, produces recalcitrant seeds that are not amenable to storage for any significant period, as they germinate rapidly upon removal from the fruit (personal observations) and are highly desiccation- and chilling- sensitive (Chin and Roberts, 1980). This Chapter reports investigations on the development of explants alternate to seeds or embryonic axes, to provide suitable specimens for cryopreservation, so facilitating long-term germplasm preservation. Experimental studies to this end involved the production of explants using two developmental pathways, organogenesis and embryogenesis. One of the main factors influencing successful cryostorage is the choice of explant, as explained in Chapter 1. Explants that are small and can be dehydrated to low water contents without loss of viability while still maintaining genetic integrity, are explants most suitable for freezing. Three types of explants were selected as having potential for the long-term preservation of cocoa germplasm, i.e. axillary shoots generated from greenhouse-derived nodal segments, nodal segments derived from *in vitro*-grown plants and somatic embryos. The main aspects of the study were first to generate these explants by establishing appropriate *in vitro* protocols, followed by facilitating the establishment of plantlets from these explants and then attempting to develop a protocol for explant cryopreservation.

2.1 Materials and Methods

2.1.1 Plant material

Cocoa fruits, commonly termed pods, that had been decontaminated after harvest with either sodium hypochlorite (NaOCl) or potassium permanganate solutions, were transported by air from Nigeria, Ghana and Cameroon. In all cases, the fruits were carried in the aircraft cabin to avoid exposure to the cold conditions of the hold (Berjak and Pammenter, 2004). Upon arrival to the laboratory, the fruits were wiped with a 2% NaOCl solution and stored at 16°C. Plants were generated via seeds in a greenhouse or by means of embryonic axes *in vitro*, both being used as stock material for subsequent studies.

2.1.2 Establishment of axillary shoots via greenhouse-derived nodal segments

2.1.2.1 Establishment of greenhouse plants

Cocoa seeds were cleaned and decontaminated in 1% calcium hypochlorite $Ca(OCl)_2$ for 5 min followed by three rinses in sterile distilled water. Seeds were then sown, three per 20 *l* plastic planting bag filled with potting soil, and maintained in a relatively sterile greenhouse environment at temperatures between 25 and 32ºC. Plants were watered twice a day, 15 min each in the morning and afternoon, via a drip irrigation system. Established plants were treated with organic nutrients (Seagrow; Starke Ayres, South Africa) and trace elements (Trelmix; Hubers, South Africa) periodically, and were sprayed once every two months with an insecticide (Kemprin; Shaik-Agchem, South Africa) during the summer months.

2.1.2.2 Decontamination of nodal segments for culture initiation

After application of NaOCl to single nodal explants (Treatment 1) was found to be unsatisfactory (see Results and Discussion) and thus multi-nodal segments from the greenhouse-grown plants were decontaminated using a two-step procedure (Treatment 2). The segments were first treated with 1% NaOCl for 10 min, subsequently trimmed to one or two node explants under sterile conditions and then decontaminated in 1% $Ca(OCl)_2$ for 3 min with three rinses in sterile distilled water after each step. After two weeks in culture, the percentage of contaminated explants was recorded before these were discarded. The nodal segments generated from greenhouse-grown plants were too large in diameter to be used for cryopreservation studies, therefore the explants selected were axillary shoots (Figure 2.1). (In the present study, after bud-break, the structure is referred to as an axillary shoot.)

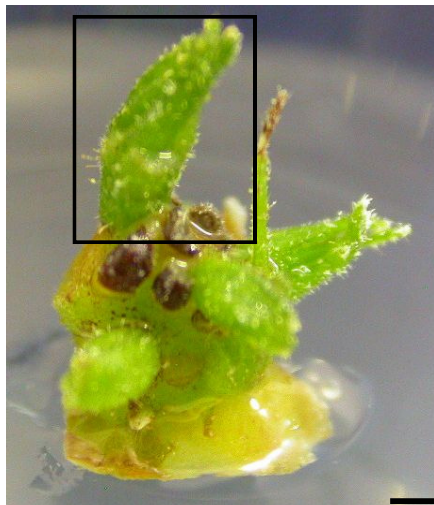


Figure 2.1: Axillary shoot selected as explant for cryopreservation, highlighted in square. Scale bar = 1 mm

2.1.2.3 Establishment of bud-break and multiplication media

Nodal segments were trimmed and decontaminated as described in 2.1.2.2. Two protocols were tested for bud-break of cocoa nodal segments initially, that established by Flynn *et al*. (1990) and Traore *et al*. (2003), respectively. The protocol established by Flynn *et al*. (1990) contained WPM (Woody Plant Medium, [Lloyd and McCown, 1984]), MS vitamins, 20 g l^{-1} sucrose, 0.5 mg l^{-1} adenine sulphate, 0.4 mg l^{-1} leucine, 0.4 mg l^{-1} arginine, 0.4 mg l^{-1} lysine, 0.2 mg l^{-1} tryptophan and 8.0 g l^{-1} Bacteriological agar (Merck, Gauteng). The protocol by Traore *et al*. (2003) contained half-strength DKW (Driver and Kuniyuki Walnut medium, [Driver and Kuniyuki, 1984]), 5.0 g l^{-1} sucrose, 10 g l^{-1} glucose, 0.3 g l^{-1} potassium nitrate, 0.3 mg l^{-1} leucine, 0.4 mg l^{-1} arginine, 0.4 mg l^{-1} lysine, 0.5 mg l^{-1} tryptophan and 4.0 g l^{-1} Gelrite. In addition, the effect of different basal media (MS, [Murashige and Skoog, 1962; Highveld Biological, South Africa], WPM and DKW) each at full- and half-strength salt concentrations, supplemented with 20 g l^{-1} sucrose and 4.0 g l^{-1} Gelrite® (Polychem, South Africa) without the inclusion of other media additives, was investigated for bud-break. Subsequently, to increase the number of shoots forming per nodal segment such that more explants (i.e. axillary shoot) would be available for cryopreservation studies, explants were positioned on WPM containing 0.1 – 1.0 mg l^{-1} 6-benzylaminopurine (BAP), 20 g l^{-1} sucrose and 4.0 g l^{-1} Gelrite. Twenty nodal segments were cultured

individually on 10 ml multiplication medium within 25 x 100 mm culture tubes. After eight weeks in culture, percentage bud-break, time taken for bud-break to occur and number of shoots forming per nodal segment, were recorded.

2.1.2.4 Rooting of shoots

After bud-break, axillary shoots elongated when attached to the parent explant in culture, were used in subsequent rooting experiments. Elongated axillary shoots, 15 - 20 mm, were placed on the basic medium, containing WPM, 20 g l^{-1} sucrose and 4.0 g l^{-1} Gelrite with naphthaleneacetic acid (NAA; 0.5, 1.0, 2.0 or 2.5 mg l^{-1}), 2,4-dichlorophenoxyacetic acid (2,4-D; 0.5, 1.0, 2.0 or 3.0 mg l^{-1}) and indole-3-butyric acid (IBA; 0.5, 1.0, 2.0 or 3.0 mg l^{-1}). In addition, following the procedure of Traore *et al.* (2003), shoots were cultured on the basic medium supplemented with 3.0 mg l^{-1} IBA for 24 h, 3 d or 7 d and subsequently transferred onto medium devoid of growth regulators to promote rooting. Twenty axillary shoots were cultured individually on 10 ml rooting medium, each within a 25 x 100 mm culture tube. After eight weeks in culture, the number of explants producing roots was assessed.

2.1.3 Pre-treatment and elongation of axillary shoots

The excision of the small axillary shoots as required for cryopreservation necessitated prior development of a regeneration protocol. Two size categories of axillary shoots were investigated; however, explants < 2 mm did not survive, therefore 2 - 4 mm explants were used in subsequent treatments. A pre-treatment and elongation step that would ultimately follow cryopreservation had to be established, such that the excised axillary shoot would establish a plantlet, since shoots shorter than 10 mm did not root. Axillary shoots, 2 - 4 mm long, were excised from the parent plant and pre-treated with an anti-oxidant solution to counteract the consequences of exudation of phenolics, prior to subsequent manipulations. The explants were treated with 0.5% ascorbic acid for 10 min followed by 5 min decontamination in $Ca(OCl)_2$ with sterile water rinses after each treatment.

The 2 - 4 mm axillary shoots initiated for elongation experiments were pre-treated and decontaminated as described above, and thereafter subjected to different treatments to

promote elongation. For elongation, MS medium, WPM and DKW medium, each at full- and half-strength salt concentrations, were assessed. The next strategy was etiolation; explants were placed in the dark for 24 h, 3 d, 7 d, 14 d or 28 d and then were transferred to a 16 h photoperiod growth room with a PPFD of 60 µmol m^{-2} s^{-1} at 25±2˚C day/23±2˚C night. Finally, to encourage elongation, axillary shoots with a segment of stem attached basally, were exposed to media containing 0.1 – 1.0 mg l^{-1} BAP. After eight weeks, percentage axillary shoot survival, shoot length and percentage explants producing callus were assessed. Twenty culture tubes (25 x 100 mm) containing 10 ml of medium were used for all experiments, with one explant/ tube.

2.1.4. Establishment of *in vitro* nodal segments for cryopreservation

2.1.4.1 Establishment of *in vitro* plantlets

Embryonic axes were excised from the seeds and decontaminated with 1% $Ca(OCl)_2$ for 10 min followed by three rinses with sterile distilled water. The axes were then germinated on MS medium supplemented with 30 g l^{-1} sucrose and 8.0 g l^{-1} bacteriological agar. Twenty axes were placed individually in culture tubes (25 x 100 mm) containing 10 ml of germination medium. From previous studies, WPM with 20 g l^{-1} sucrose and 4.0 g l^{-1} Gelrite, had been established as the best maintenance medium for *in vitro* established plantlets (Naidoo, 2004). Plantlets were subcultured every 4 - 6 weeks on maintenance medium in Magenta boxes (Highveld Biological, South Africa). Two to three plantlets per Magenta box were maintained on 80 ml maintenance medium.

2.1.4.2 Shoot multiplication via embryonic axes

In order to bulk up the stock material for subsequent cryopreservation studies using *in vitro* nodal segments, the embryonic axes were cultured on either a gelled medium or maintained transiently (see below) in a RITA® (Recipient of Automated Temporary Immersion; PALL, Gelman Laboratory, France) system. The axes were excised from the seeds and decontaminated as described in 2.1.4.1. Following decontamination, twenty axes were cultured individually in 25 mm x 100 mm culture tubes on 10 ml germination medium containing 0.1, 1.0, 5.0 or 10 mg l^{-1} BAP or 2-isopentenylamino

purine (2-iP) for four weeks. Thereafter, four axes were placed on 80 ml hormone-free WPM medium with 20 g l^{-1} sucrose and 4.0 g l^{-1} Gelrite in individual Magenta boxes.

The RITA system was used to pulse newly excised axes with MS liquid medium containing 30 g l^{-1} sucrose and 0.1, 1.0 or 5.0 mg l^{-1} of BAP or 2-iP using three different flush intervals (30 sec flush and 30 min rest; 1 min flush and 1 h rest; 1 min flush and 3 h rest). There were 15 axes/vessel with 200 ml liquid multiplication medium. The axes were incubated in the vessels for 72 h, after which three axes were transferred to each of five individual Magenta boxes, containing 80 ml maintenance medium devoid of plant growth regulators. After eight weeks in culture, percentage germinants, number of shoots formed per explant as well as the length of the shoots, was recorded.

2.1.4.3 Establishment of bud-break medium for *in vitro* nodal segments

Nodal segments were excised from 8 – 12 weeks-old *in vitro* plants. The nodal segments were trimmed to 1 - 2 node explants, which were pre-treated with 0.5% ascorbic acid for 10 min and decontaminated with 1% $Ca(OCl)_2$ for 5 min followed by three rinses in sterile distilled water (as explained in 2.1.3, for axillary shoots). Bud-break medium for *in vitro* nodal segments consisted of half-strength DKW medium containing 10 g l^{-1} glucose, 5.0 g l^{-1} sucrose, 0.3 g l^{-1} potassium nitrate, 0.4 mg l^{-1} arginine, 0.3 mg l^{-1} leucine, 0.4 mg l^{-1} lysine, 0.5 mg l^{-1} trytophan and 4.0 g l^{-1} Gelrite (Traore *et al*., 2003). In an attempt to reduce the time required for bud-break to occur, another set of nodal segments was placed on the same bud-break medium supplemented with 0.1 – 1.0 mg l^{-1} BAP. One nodal segment was introduced per 25 x 100 mm tube containing 10 ml of medium, with each treatment utilising 20 nodal segments. After eight weeks in culture, percentage bud-break, time taken for bud-break to occur and number of axillary shoots/nodal segment were recorded.

After bud-break, axillary shoots were elongated while still attached to the *in vitro* nodal segment and rooting was induced as for axillary shoots derived from greenhouse nodal segments (section 2.1.2.4, above).

2.1.5 Cryopreservation (cryo) studies using *in vitro* nodal segments

2.1.5.1 Optimisation of explant size for cooling

In vitro nodal segments were pre-treated with ascorbic acid and decontaminated with $Ca(OCl)_2$ as described in 2.1.4.3. Three different sizes of explant, 2 - 3 mm, 4 - 5 mm and 6 - 7 mm were placed on half-strength DKW medium incorporating 10 g l^{-1} glucose, 5.0 g l^{-1} sucrose, 0.3 g l^{-1} potassium nitrate, 0.4 mg l^{-1} arginine, 0.3 mg l^{-1} leucine, 0.4 mg l^{-1} lysine, 0.5 mg l^{-1} trytophan and 4.0 g l^{-1} Gelrite, to obtain the optimal size explant that would withstand subsequent desiccation and cryogen immersion. To optimise bud-break, the effects of different culture vessels viz. 65-mm and 90-mm Petri dishes and 25 x 100 mm culture tubes, were tested. Five explants were plated per Petri dish and one explant introduced into each culture tube.

2.1.5.2 Desiccation of nodal segments for subsequent cooling

Nodal segments (6 - 7 mm) were desiccated via physical and osmotic methods. Physical desiccation was carried out by placing explants in a flash-drying apparatus (Pammenter *et al*., 2002) for 10, 20, 30, 40, 60, and 80 min, or on a piece of sterile filter paper in an air- tight culture bottle over 50 g of activated silica gel for 1 - 6 h. Osmotic dehydration was achieved by placing explants on half-strength DKW medium incorporating increasing sucrose concentrations (0.3 M, 0.5 M, 0.75 M, 1.0 M) for 24 h incubation periods at each sucrose concentration. After each desiccation period, the water content of five nodal segments was determined gravimetrically after oven-drying at 80˚C for 48 h, and expressed on a dry mass basis (g g^{-1} dmb). To assess survival after dehydration, at each desiccation interval, 15 nodal segments were placed individually in 25 x 100 mm culture tubes containing 10 ml of bud-break medium. After eight weeks in culture, percentage bud-break, time taken for bud-break to occur and number of buds/nodal segment were recorded.

2.1.5.3 Sucrose pre-culture of *in vitro* nodal segments

Newly excised nodal segments (6 - 7 mm) were pre-treated as described in 2.1.4.3. Explants were then placed on bud-break medium supplemented with different concentrations (0.3 M, 0.5 M, 0.75 M, and 1.0 M) of sucrose for 24 h or 3 d. After the

respective incubation period, explant water content was determined and viability retention was assessed as described in 2.1.5.2.

2.1.6 Cryopreservation techniques

2.1.6.1 Vitrification

Nodal segments (6 - 7 mm) were pre-treated by ascorbic acid immersion and $Ca(OCl)_2$ decontamination as described in 2.1.4.3. Explants were subjected to 24 h pre-culture on half-strength DKW salts, 0.3 M sucrose, 0.3 g l^{-1} potassium nitrate, 0.3 mg l^{-1} leucine, 0.4 mg l^{-1} arginine, 0.4 mg l^{-1} lysine, 0.5 mg l^{-1} tryptophan and 4.0 g l^{-1} Gelrite. Thereafter, explants were treated with 20 ml loading solution (half-strength DKW salts, 2 M glycerol and 0.4 M sucrose) for 20 min. Explants were subsequently transferred to 2 ml cryovials and treated with the plant vitrification (PVS2) solution (Sakai, 1985), comprising half-strength DKW salts, 15 ml ethylene glycol, 15 ml dimethylsulphoxide (DMSO), 30 ml glycerol and 13.7 g sucrose, made up to 100 ml with distilled water, at either 25 or 0˚C. Explants were incubated in PVS2 solution for 10, 15, 20, 25 or 30 min within cryovials. After the incubation period, the PVS2 solution was removed, the cryovials were clipped into cryocanes which were plunged into liquid nitrogen (-196˚C). After each treatment before immersion in liquid nitrogen, a sub-sample of explants was removed for water content determination and viability assessment, as described in 2.1.5.2. Cryovials were kept in liquid nitrogen for 24 h and thereafter removed from the holding Dewar vessel (cryo tank) and the specimens thawed by plunging the cryotubes into a water bath at 40˚C for 2 min. Thereafter, explants were placed in 20 ml unloading solution (half-strength DKW salts and 1.2 M sucrose) for 20 min at room temperature. All procedures were carried out under sterile conditions. Individual nodal segments were introduced into 25 x 100 mm culture tubes containing 10 ml of bud-break medium and placed in the dark at 25±2˚C for 5 d, after which they were maintained in a growth room with a PPFD of 60 µmol m^{-2} s^{-1} at 25±2˚C day/23±2˚C night. After eight weeks in culture percentage bud-break, time taken for bud-break to occur and number of buds/nodal segment, were recorded.

2.1.6.2 Two-stage cooling

Nodal segments (6 - 7 mm) were pre-treated by ascorbic acid immersion and $Ca(OCl)_2$ decontamination as described in 2.1.4.3. Explants were then subjected to one of six pre-treatments before being placed in a freezing container, Mr Frosty® (Nalgene) (Figure 2.2), which facilitates a cooling rate of 1˚C min^{-1}, verified by the use of a thermocouple. Five explants per 2 ml cryovial were cooled to -40˚C using this apparatus in which the cryovials were bathed in isopropanol and the whole apparatus held in a -70˚C freezer for 62 min. Six pre-treatments were investigated (Table 2.1) prior to freezing, either with or without the use of cryoprotectants (glycerol alone, or combined with sucrose). For the cryoprotection experiments, explants were treated with 20 ml of 5% cryoprotectant solution for 15 min followed by a further 15 min in a 10% solution. Water content and viability assessment of nodal segments was carried out as described above (2.1.5.2). After the cooling period using Mr Frosty, the cryovials were rapidly inserted into cryocanes and plunged into liquid nitrogen. Cryovials were kept in liquid nitrogen for 1 h, after which the explants were thawed in the cryovials, in a 40˚C water bath for 2 min and then rehydrated.

Figure 2.2: **Freezing container, Mr Frosty, used for two-stage cooling. Scale bar = 10 mm**

Table 2.1: The pre-treatments performed prior to slow cooling.

Treatment	Description
a	no treatment (control)
b	pre-culture on 0.3 M sucrose for 24 h
c	pre-culture on 0.3 M sucrose for 24 h and subsequently cryoprotection with 5% glycerol for 15 min followed by 10% glycerol for 15 min
d	pre-culture on 0.3 M sucrose for 24 h and subsequently cryoprotection with 5% sucrose + glycerol for 15 min followed by 10% sucrose + glycerol for 15 min
e	cryoprotection with 5% glycerol for 15 min followed by 10% glycerol for 15 min
f	cryoprotection with 5% sucrose + glycerol for 15 min followed by 10% sucrose + glycerol for 15 min.

2.1.6.3 Rehydration of *in vitro* nodal segments after cooling

Three strategies prior to *in vitro* culturing were tested: no rehydration or decontamination, decontamination but no rehydration, and rehydration in a 1:1 solution of 1.0 µM $CaCl_2.2H_2O$: 1.0 mM $MgCl_2.6H_2O$ (CaMg solution; Mycock, 1999) for 20 min followed by decontamination. Explants that had been cryoprotected were first unloaded by soaking in a 5% cryoprotectant solution, appropriate to the cryoprotectant pre-treatment, for 15 min and then subjected to all the rehydration strategies described above. All rehydration steps were carried out in the dark, as light has been shown to exacerbate post-cryo damage suggestedly via free radical generation (Mayaba *et al*., 2002). After rehydration, nodal segments were introduced into 25 x 100 mm culture tubes with 10 ml of bud-break medium, and placed in the dark at 25±2˚C for 5 d, after which explants were maintained in a growth room with a PPFD of 60 µmol m^{-2} s^{-1} at 25±2˚C day/23±2˚C night. After eight weeks in culture percentage bud-break and contamination, were recorded.

Culture media were adjusted to pH 5.6 - 5.8 prior to autoclaving at 100 Kpa at 121˚C for 20 min for all experiments. All cultures were maintained in the growth room under a

16-h photoperiod at a PPFD of 60 µmol m^{-2} s^{-1} (above and lateral lighting) provided by fluorescent tubes at temperatures of 25±2˚C day/23±2˚C night, unless otherwise stated.

2.1.7 Somatic embryogenesis via leaf and cotyledon explants

Leaf material used was obtained from greenhouse-grown plants and cotyledon material from mature seeds. Leaf material was decontaminated using a two-step procedure; first, immersion for 10 min in 1% NaOCl followed by three rinses in sterile distilled water, followed by trimming to 10 mm x 10 mm squares under sterile conditions, after which the explants were soaked in 1% CaOCl for 5 min followed by three rinses in sterile distilled water. Because of their high content of phenolics, cotyledon explants were initially soaked in 2% citric acid for 10 min. Thereafter, cotyledon segments were decontaminated by soaking in 70% ethanol (EtOH) for 2 min followed by 10 min in 2% Hibitane® (active ingredient, chlorhexidine gluconate, Dismed Pharma, South Africa) and a further 10 min in 1% NaOCl with three rinses in sterile distilled water after each treatment. Leaf and cotyledon explants were then placed on two different basal media, MS and DKW, each supplemented with 0.5, 1.0, 2.0, 3.0, or 5.0 mg l^{-1} 2,4-D alone; 0.5 or 1.5 mg l^{-1} 2,4-D in combination with 0.1, 0.5 or 1.0 mg l^{-1} BAP; or 1.0, 10, 50 or 100 mg l^{-1} 2-iP; or 0.01, 0.05, 0.1 or 1.0 mg l^{-1} thidiazuron (TDZ), to induce embryogenic callus. Explants were incubated for six weeks, after which specimens with embryogenic callus were subcultured onto WPM supplemented with 0.1, 0.5 or 1.0 mg l^{-1} BAP or kinetin. After four weeks, the explants were subcultured onto DKW medium devoid of growth regulators for a further four weeks. All explants were incubated in the dark at 25±2˚C. Five to seven explants were plated in 90-mm Petri dishes each containing 30 ml of medium. Cultures were screened after each treatment and the percentage of explants producing callus, the type of callus produced as well as the amount of each callus type forming per explant was qualitatively assessed. The amount of callus produced was scored by using (+) symbols, one symbol representing quarter of the explant. Therefore, four (+) symbols per treatment meant the entire upper surface of the explant was covered with callus of the respective type.

2.1.8 Photography and Microscopy

All photographs were taken with a Nikon Coolpix® 4500 digital camera. Both embryogenic and non-embryogenic callus was spread on microscope slides, stained with a 0.1% Toluidine Blue solution made up in phosphate buffered saline (PBS) (pH 7.4), viewed and images captured using a Nikon Biphot® photomicroscope.

2.1.9 Data analysis

Statistical analyses were performed using SPSS 15.0 for Windows. The Student's-t test was used for two sample analysis and Scheffé's multiple range test (one-way ANOVA) for three and more sample analyses that displayed parametric data. The chi-squared test was used for non-parametric binomial data, to assess for significant differences within a table of results where percentages obtained were compared with each other. The level of significance for all statistical analyses performed was taken to be $p < 0.05$. The mean value for each treatment was assigned an alphabetical symbol. Mean values that did not share the same letter are recognised as being significantly different.

2.2 Results and Discussion

2.2.1 Establishment of axillary shoots

The establishment of axillary shoots as possible explants for cryopreservation was investigated for nodal segments derived from young greenhouse-grown plants. Seeds potted in the greenhouse germinated successfully giving rise to stock plants for subsequent experiments. The plants in the greenhouse (constructed with an ante-chamber at the entrance) were maintained in a relatively sterile environment (precluding extraneous insects and most contaminants), so providing a reasonably clean source of primary explant material for *in vitro* studies.

2.2.1.1 Decontamination of nodal segments for culture initiation

Contamination is a major problem to overcome when initiating explants, especially when using plant material from field conditions. The decontaminant used for nodal segments was NaOCl, which had previously been used for nodal segments of cocoa (Flynn *et al*., 1990), and for example, for those of *Guadua angustifolia* (Jiménez *et al*., 2006) and *Quercus euboica* (Kartsonas and Papafotiou, 2007). Certain decontaminants can cause tissue death if the application period is prolonged. Therefore, the choice of decontaminant is important and the time of application should be optimised such that the treatment is effective in removing contaminants while causing minimum injury to the tissue. The decontamination of cocoa nodal explants via a two-step procedure (Treatment 2) was more successful in eliminating bacterial contamination, compared with a single decontaminant (Treatment 1) (Table 2.2). The cut surfaces of single nodal explants browned when treated with 1% NaOCl (Treatment 1), an effect that was avoided when decontamination with 1% NaOCl was carried out before trimming of (multi-nodal) explants. This procedure (Treatment 2) protected the base of the segments to be cultured. Explants from Treatment 1, were first trimmed to a length of 15 mm and then subjected to 1% NaOCl for 10 min with three rinses in sterile water after decontamination. In addition to the browning, 30% of explants remained contaminated (Table 2.2). The two-step procedure (Treatment 2), which was routinely used after the initial trials, reduced contamination to 10% and only a few explants browned after decontamination even though all had been immersed in 1% CaOCl for 3 min after trimming. (Although application of the Chi-squared test did not indicate the 30% to

10% reduction in contamination to be significant, reduction in browning was significant [$p < 0.05$].) Calcium hypochlorite is considered to be more stable and less injurious than NaOCl (Anon, 2008), which was evident during this study.

Other investigators have reported favourably on similar use of multi-nodal segments as the starting material. For example, Thakur and Sood (2006) developed an efficient method for explant decontamination for elongated internodes of bamboo, tea and rose. Those authors tested the effects on contamination and shoot proliferation of decontaminating multi-node explants or single node explants. They showed that trimming of explants prior to decontamination resulted in deep penetration of the decontaminants into the tissues, causing long-lasting toxic effects which resulted in delayed and reduced growth responses. In addition, Thakur and Sood (2006) reported that explants trimmed for culture initiation prior to decontamination showed higher contamination percentages than those trimmed down after decontamination, which was consistent for all species they investigated. Results obtained for cocoa nodal segments in this study concur with the findings of Thakur and Sood (2006).

In this study, all experiments performed using greenhouse-grown nodal segments, utilised non-lignified, and newly emerged axillary shoot material since mature tissues have been shown to be less responsive in culture (e.g. George, 1996; Hartmann *et al.*, 2001). More recently, for example, Kartsonas and Papafotiou (2007) showed that juvenile tissue of *Quercus euboica* had higher multiplication rates than explants from adult plants.

Table 2.2: Effect of two different decontamination procedures on nodal segments from greenhouse-derived plants. Treatment 1: single nodal segments were trimmed to 15 mm and then immersed in 1% NaOCl for 10 min followed by three rinses in sterile water. Treatment 2: multi-nodal segments were first treated with 1% NaOCl for 10 min, then trimmed to 15 mm explants and subsequently decontaminated for 3 min in 1% $Ca(OCl)_2$ with three rinses in sterile distilled water after each step. n=20-25. Mean values represented by the same alphabetical letters are not significantly different (Chi-squared test, $p < 0.05$).

Treatment No.	Decontaminants	Treatment duration (min)	% contamination	% explant browning
1	1% NaOCl	10	30 [a]	50 [a]
2	1% NaOCl 1% $Ca(OCl)_2$	10 3	10 [a]	10 [b]

2.2.1.2 Development of bud-break and multiplication media

The proliferation of buds from nodes along the shoot below the apical meristem can be simply achieved in some species while special stimuli are required for other species. Many factors affect bud-break, e.g. explant age and origin (Kartsonas and Papafotiou, 2007), seasonal influence (Mishra *et al.*, 2007), medium composition (Traore *et al.*, 2003), as well as the incorporation of growth regulators into the culture medium (Chen *et al.*, 2006; He *et al.*, 2007; Sanatombi and Sharma, 2007).

Two established protocols for bud-break in cocoa nodal segments were investigated to evaluate the response of material derived from greenhouse-grown plants in this study. These were taken from Flynn *et al.* (1990), who experimented on non-woody, 40 - 80 mm cocoa nodal segments derived from field-grown material, and Traore *et al.* (2003) who used explants from plantlets derived from somatic embryos.

In this study, the percentage of explants producing axillary shoots was relatively high, with no significant difference between results using the protocols of Flynn *et al.* (1990) and Traore *et al.* (2003) (Table 2.3). However, only one shoot formed per explant

irrespective of the protocol, with no multiplication occurring, although, there was a significant difference in the length of shoots four weeks after bud-break. Axillary shoots produced via the protocol of Traore *et al.* (2003) were significantly longer in comparison with those using the method of Flynn *et al.* (1990) (Table 2.3). The time taken for bud-break was the same for both protocols. An attempt was then made to reduce the time required for bud-break to occur, by the use of different, and different concentrations, of basal media, without the inclusion of amino acids or other additives (Table 2.4).

Table 2.3: Axillary bud-break using nodal segments from greenhouse-established plants. Comparing the WPM based protocol of Flynn *et al*. (1990) and that of Traore *et al*. (2003) based on DKW medium. n=20. Mean values represented by the same alphabetical letters within each column are not significantly different (Student's-t test and Chi-squared test, $p < 0.05$).

Medium according to:	% explants producing shoots	Avg. no. of shoots/explant	Avg. shoot length (mm)	Time taken for bud-break (days)
Flynn *et al.* (1990)	60 [a]	1 [a]	4.1 [a]	28
Traore *et al.* (2003)	70 [a]	1 [a]	5.9 [b]	28

The use of different basal media and at different concentrations influenced the percentage of explants producing axillary shoots as well as the time required for bud-break to occur (Table 2.4). There was a significant difference between the number of explants producing axillary shoots, 100% on full MS and WPM, and as few as 50% on the medium based on full-strength DKW salts.

Table 2.4: Effect of different basal media on axillary bud-break using greenhouse-derived nodal segments. Three different basal media were tested, MS - Murashige and Skoog, 1962, WPM - Woody Plant Medium, Lloyd and McCown, 1981 and DKW - Driver and Kuniyuki, 1984 each supplemented with 20 g l^{-1} sucrose and 4.0 g l^{-1} Gelrite. n=20. Mean values represented by the same alphabetical letters within each column are not significantly different (Chi-squared and Scheffé's multiple range test, $p < 0.05$).

Medium based on:	% explants producing buds	Avg. no. of buds/explant	Avg. shoot length (mm)	Time taken for bud-break (days)
MS	100 ^{a}	1 ^{a}	4.3 ^{a}	28
½ MS	75 ^{b}	1 ^{a}	8.1 ^{b}	21
¼ MS	80 ^{b}	1 ^{a}	3.8 ^{a}	28
WPM	100 ^{a}	1 ^{a}	7.7 ^{b}	17
½ WPM	80 ^{b}	1 ^{a}	4.6 ^{a}	25
DKW	50 ^{b}	1 ^{a}	3.4 ^{a}	28
½ DKW	75 ^{b}	1.1 ^{a}	3.7 ^{a}	28

To determine the best medium for bud-break, the time taken for this to occur was assessed. Bud-break occurred, after 17 days on WPM, compared with 28 days on MS medium. The advantage of bud-break occurring more quickly, is that it enables the production of many axillary shoots in a shorter period. Thus, in this study, WPM supplemented with 20 g l^{-1} sucrose and 4.0 g l^{-1} Gelrite was used for subsequent investigations. In addition, the cost of establishing axillary shoot cultures was reduced since the necessity for incorporation of other media additives as used in the protocols of Flynn *et al*. (1990) and Traore *et al*. (2003) for cocoa, was eliminated.

The gelling agent used in culture media can affect tissue growth (Saadat and Hennerty, 2002). Gelrite® was used in the present experiments instead of Bacto agar originally used in the protocol developed by Flynn *et al*. (1990) for bud-break of cocoa nodal segments. Gelrite (and Phytagel®) are trade names for gellan gum preparations and are currently widely used for different tissue culture systems. Use of Gelrite as the

solidifying agent could have been at least partly responsible for the success achieved in obtaining 100% bud-break for cocoa nodal segments in this study as well as the shorter time frame (17 d on WPM) required for bud-break to occur. In contrast, in the experiments of Flynn *et al*. (1990) bud-break occurred in 85% of cases, taking 20 – 24 days to form an axillary shoot. The present finding concurs with a study performed on *Juglans regia* (Persian walnut) which showed Gelrite to be superior in culture compared with Bacteriological agar by facilitating significantly greater callus fresh weight, shoot fresh weight, main shoot length and leaf number per shoot (Saadat and Hennerty, 2002) and this has also been shown in other investigations (Pasqualetto *et al*., 1988; Nairn *et al*., 1995).

Proliferation and multiplication of axillary shoots from nodal segments is a common micropropagation protocol used for commercial crop species (Wilhelm, 2003). For the multiplication of axillary shoots using cocoa nodal segments, two bud-break media were selected, viz. WPM (Medium 1) and that based on half-strength DKW medium, according to the protocol established by Traore *et al*. (2003) (Medium 2). The highest multiplication rate was achieved on half-strength DKW medium (Medium 2) supplemented with 1.0 mg l^{-1} BAP, where an average of 2.9 shoots per explant was formed (Figure 2.3). This is in agreement with investigations on other species that reported either 0.5 or 1.0 mg l^{-1} BAP to be optimal for the multiplication of axillary shoots (Saadat and Hennerty, 2002; Chen *et al*., 2006; Matu *et al*., 2006; Ascough *et al*., 2007; Bruno *et al*., 2007).

In addition, the inclusion of BAP in the culture media in the current investigation reduced the time required for bud-break to occur; the inclusion of 0.1 or 0.5 mg l^{-1} BAP to WPM reducing the time required for bud-break to 14 days (cf Tables 2.4 and 2.5). Shoot length was not significantly affected by either medium or by the addition of BAP to the culture medium.

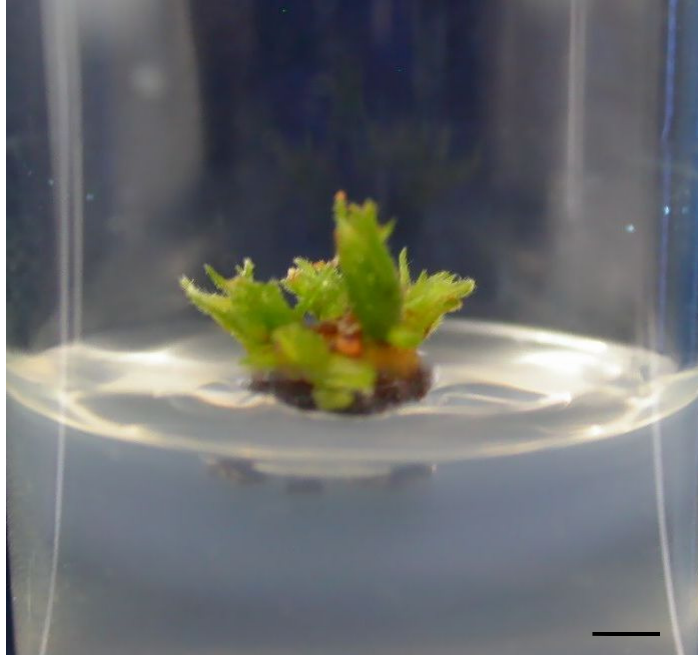


Figure 2.3: Axillary shoot multiplication achieved on DKW medium supplemented 1.0 mg l^{-1} BAP. Scale bar= 3 mm

Table 2.5: Axillary shoot multiplication via nodal segments from greenhouse-grown plants, using BAP. Media 1 and 2: WPM and half-strength DKW salts, respectively, supplemented with various concentrations of BAP. In all cases, media were solidified with Gelrite. n=20. Mean values represented by the same alphabetical letters within each column are not significantly different (Chi-squared test and Scheffé's multiple range test, $p < 0.05$).

Medium	[BAP] (mg l^{-1})	% explants producing shoots	Avg. no. of shoots/explant	Avg. shoot length (mm)	Time taken for bud-break (days)
1	0.1	95 ^{a}	2.0 ^{ab}	3.3 ^{a}	14
	0.5	95 ^{a}	1.8 ^{ab}	3.9 ^{a}	14
	1.0	75 ^{a}	2.0 ^{ab}	3.2 ^{a}	17
2	0.1	80 ^{a}	1.3 ^{a}	3.3 ^{a}	21
	0.5	80 ^{a}	2.0 ^{ab}	3.4 ^{a}	21
	1.0	80 ^{a}	2.9 ^{b}	4.2 ^{a}	21

2.2.1.3 Rooting of axillary shoots

Three auxins were investigated for their ability to induce rooting of *in vitro* cocoa shoots, *viz*. NAA, 2,4-D and IBA. No concentrations of 2,4-D tested facilitated rooting, while very low rooting percentages were achieved when NAA was added to the culture medium. The best result was obtained using medium supplemented with 3.0 mg l^{-1} IBA (Table 2.6), when 40% of explants rooted. Studies performed on a variety of other species have also shown success in rooting of shoots when IBA has been incorporated into the culture medium (Ascough *et al*., 2007; Dhooghe and Labeke, 2007, He *et al*., 2007; Sanatombi and Sharma; 2007).

Table 2.6: Effect of NAA, 2,4-D and IBA on the rooting capacity of axillary shoots. Axillary shoots, 15 - 20 mm long were excised from nodal segments and placed on WPM, 20 g l^{-1} sucrose and different concentrations of, 2,4-dichlorophenoxyacid (2,4-D), 1-naphthalene acetic acid (NAA) and indole butyric acid (IBA). n=15. Mean values represented by the same alphabetical letters are not significantly different (Chi-squared test, $p < 0.05$).

Growth regulator concentration (mg l^{-1})			% explants producing roots
NAA	2,4-D	IBA	
0.5	-	-	0^{a}
1.0	-	-	5^{ab}
2.0	-	-	10^{ab}
2.5	-	-	10^{ab}
-	0.5	-	0^{a}
-	1.0	-	0^{a}
-	2.0	-	0^{a}
-	3.0	-	0^{a}
-	-	0.5	0^{a}
-	-	1.0	0^{a}
-	-	2.0	20^{b}
-	-	3.0	40^{c}

A previous study carried out on cocoa showed a significant increase in rooting of explants to occur when cocoa shoots were cultured on medium containing 3.0 mg l^{-1} IBA for various periods, with subsequent transfer onto a medium devoid of growth regulators (Traore *et al*., 2003). In the present study, this approach significantly increased rooted shoots to 70% (Table 2.7). More shoots developed roots over an incubation period of 7 d on medium supplemented with 3.0 mg l^{-1} IBA compared with incubation periods of 24 h and 3 d. This method has also been used for shoots of *Quercus euboica*, where incubation on medium containing 9.84 µM IBA for one week and then transferred onto hormone-free medium gave the best percentage of rooted shoots (Kartsonas and Papafotiou, 2007). Matu *et al*. (2006) achieved successful rooting

for *Maytenus senegalensis* via a two-stage process where shoots were first treated for 120 h with a liquid medium containing 25 mg l^{-1} IBA in the dark and subsequently transferred to a hormone-free medium under a 12 h photoperiod.

Table 2.7: Effect of 3.0 mg l^{-1} IBA on the rooting capacity of axillary shoots for different incubation periods. Axillary shoots, 15 - 20 mm long were incubated for 24 h, 3 d and 7 d on WPM, 20 g l^{-1} sucrose and 3.0 mg l^{-1} of indole butyric acid (IBA), and then placed on WPM devoid of IBA. n=20. Mean values represented by the same alphabetical letters are not significantly different (Chi-squared test, $p < 0.05$).

Incubation period	% explants producing roots
24 h	0 ^{a}
3 d	20 ^{a}
7 d	70 ^{b}

2.2.2 Axillary shoots as explants for cryopreservation

2.2.2.1 Optimisation of explant size and pre-treatment for cooling

The optimisation of the explant size is a critical factor in initial dehydration followed by cooling for cryopreservation (Baek *et al*., 2003). Explants that are large, particularly in diameter, will require longer dehydration periods since they have low surface area: volume ratios. The smaller the explant, the faster the cooling rate attainable, and the better the chances both of successful dehydration and cooling, both of which should be rapid for desiccation- sensitive specimens (e.g. Pammenter *et al*., 2002). However, explants that are too small may lack the capacity for further development, either inherently, or because of excision injury. In this study, axillary shoots that were smaller than 2 mm in length did not survive while axillary shoots that were 2 - 4 mm long had very low survival percentages when explants were excised from greenhouse-grown nodal segments and placed on WPM to assess survival and contamination (Table 2.8).

Table 2.8: Optimisation of axillary shoot size for cryopreservation. Axillary shoots were excised and divided into two classes, < 2 mm and 2 - 4 mm and, after decontamination were placed on WPM, 20 g l^{-1} sucrose, 4.0 g l^{-1} Gelrite, devoid of plant growth regulators. n=30. Mean values represented by the same alphabetical letters within each column are not significantly different (Chi-squared test, $p < 0.05$).

Axillary shoot size	% shoot survival	% explant browning	% contamination
< 2 mm	0 [a]	83 [a]	20 [a]
2 - 4 mm	20 [b]	60 [a]	20 [a]

To facilitate *in vitro* growth and expansion of the axillary shoots, different growth regulators in a range of concentrations were incorporated into WPM as follows: 0.1, 0.5, 1.0, 2.0, 5.0 or 10 mg l^{-1} GA_3 or kinetin, or 0.1, 0.5, 1.0, 2.0 or 3.0 mg l^{-1} BAP, or 0.1 or 0.5 mg l^{-1} NAA in combination with either 1.0, 2.0 or 3.0 mg l^{-1} kinetin. Irrespective of the plant growth regulator supplementation, all axillary shoots became necrotic (results not shown). Different culture vessels *viz*: 90-mm Petri dishes, culture tubes and Magenta boxes were also tested using WPM devoid of growth regulators. The same explant response, i.e. tissue death (results not shown) was apparent in all the culture vessels.

Axillary shoots excised from the nodal segment in a previous study on cocoa showed a similar response, i.e. failure to grow in culture, with a positive correlation between explant size and growth response (Flynn *et al*., 1990). The injury caused to explants upon excision from maternal tissue usually causes a wounding response, often in the form of phenolics production (George, 1996). The reaction is chain linked, where cells from the site of injury initially become brown and this reaction progressively spreads through the tissue, until finally, the entire explant dies. In an attempt to investigate the response of excised axillary shoots to anti-oxidant solutions that are known to reduce or hinder the exudation of phenolics, axillary shoots (2 - 4 mm) were treated with two anti-oxidant solutions, citric plus ascorbic acids, or ascorbic acid alone, for varying periods (Table 2.9).

It appeared that phenolics produced by the excised axillary shoot were associated with explant browning and ultimately death, since exposure of axillary shoots to the anti-oxidant solutions significantly increased explant survival. The combination of ascorbic and citric acid resulted in an increase in shoot survival with increasing treatment duration, while treatment with 0.5% ascorbic acid alone resulted in the highest percentage of shoot survival, 90%, after a 10-min treatment period (Table 2.9). As this treatment resulted in significantly lower explant browning, it was selected as the best pre-treatment. Contamination that occurred was eliminated in subsequent experiments by soaking explants in 1% $Ca(OCl)_2$ for 5 min following ascorbic acid treatment.

Table 2.9: Effect of anti-oxidant solutions used for pre-treatment of axillary shoots to reduce phenolics production. Axillary shoots used were 2 - 4 mm long and were placed on WPM supplemented with 20 g l^{-1} sucrose, 4.0 g l^{-1} Gelrite devoid of growth regulators. n=20. Mean values represented by the same alphabetical letters within each column are not significantly different (Chi-squared test, $p < 0.05$).

Treatment	Time (min)	% shoot survival	% explant browning	% Contamination
1% Citric acid + 1% Ascorbic acid	5	20^{a}	60^{b}	10^{abc}
	10	40^{ab}	30^{ab}	30^{c}
	20	50^{ab}	40^{b}	10^{abc}
	30	50^{ab}	30^{ab}	20^{bc}
0.5% Ascorbic acid	5	50^{ab}	30^{ab}	0^{a}
	10	90^{c}	5^{a}	5^{ab}
	20	80^{bc}	20^{ab}	0^{a}
	30	60^{b}	30^{ab}	10^{abc}

2.2.2.2 Elongation of axillary shoots

Excision of small axillary shoots requires an intermediate elongation step before the explant can be rooted in order to establish a plantlet. Following the anti-oxidant treatment, excised axillary shoots were subjected to different treatments to achieve elongation. Due to the limited time available for this study and the large number of

explants used in previous treatments, only three treatments were tested in the elongation trials. The use of different basal media was tested, since the composition of the basal medium has been shown to influence shoot growth (Saadat and Hennerty, 2002). Axillary shoot survival was not significantly affected by the use of different basal media ($p > 0.05$), while shoot length was affected ($p < 0.05$, Table 2.10). The use of half-strength WPM resulted in minimal shoot growth while the medium established by Traore *et al.* (2003) for bud-break resulted in significantly longer shoots (Table 2.10). The basal medium used in that protocol is DKW medium which was established for walnut shoot culture by Driver and Kuniyuki (1984). This medium is similar to MS in its nitrogen content but contains higher concentrations of calcium, sulphur and magnesium (Saadat and Hennerty, 2002; Tan and Furtek, 2003). Murashige and Skoog and DKW medium are high salt media as compared with WPM which is a low salt medium, and which was found not to be appropriate for Persian walnut shoot culture (Saadat and Hennerty, 2002). Similarly, half-strength WPM in this study yielded the least shoot elongation. This could have been a result of the limitation of salts as well nitrogen in the medium, since cacao trees naturally require soil rich in nutrients and nitrogen to facilitate growth (Anon, 2002).

Although the shoot length was significantly different from the other treatments with the use of half-strength DKW salts as in the protocol of Traore *et al*. (2003), it was not adequate to facilitate rooting of the explants, none that were less than 10 mm long producing roots (results not shown). The formation of callus on the cut surface of the shoot was minimal with explants on only MS, while those on half-strength MS and half-strength WPM produced callus (Table 2.10), thus was not a limiting factor. The proliferation of callus at the base of the tissue could reduce explant growth as well as limit the availability of nutrients to the explant (George, 1993).

Table 2.10: Different basal salts used to promote elongation of excised, 2 - 4 mm long, axillary shoots. Comparing WPM, MS based medium and the protocol of Traore *et al.* (2003) based on DKW medium. n=20. Mean values represented by the same alphabetical letters within each column are not significantly different (Chi-squared and Scheffé's multiple range test, $p < 0.05$).

Basal salt	% shoot survival	Shoot length (mm)	% explants producing callus
WPM	80 [a]	3.7 [bc]	0 [a]
½ WPM	60 [a]	2.4 [a]	10 [ab]
MS	65 [a]	2.8 [ab]	10 [ab]
½ MS	70 [a]	3.1 [abc]	20 [b]
¼ MS	75 [a]	3.5 [abc]	0 [a]
Traore *et al*. (2003)	85 [a]	4.0 [c]	0 [a]

Incubation of explants in the dark causes etiolation, which enhances stem elongation and reduces leaf and root development (e.g. Salisbury and Ross, 1992; Serala *et al*., 2007). Both light and dark conditions have been found to have either positive or negative effects in shoot regeneration and organogenesis of different plant tissues (Reuveni and Evenor, 2007). Two media types were selected from those listed in Table 2.10 i.e. WPM and the protocol established by Traore *et al.* (2003) using half-strength DKW salts. Axillary shoots were incubated for 24 h, and 3, 7, 14 and 28 d in the dark on both media types. There was no significant difference in shoot survival across the treatments ($p > 0.05$, Table 2.11). However, shoot length was significantly the highest when explants were placed in the dark for 28 days achieving a length of 6.3 mm after 8 weeks in culture. Explants incubated in the dark for long periods formed callus, but, even at their longest, the axillary shoots appeared to be inadequate for root formation.

Table 2.11: The effect of etiolation on axillary shoot elongation. Medium 1 and 2: WPM and half-strength DKW salts, respectively. Axillary shoots 2 - 4 mm long were incubated in the dark for varying times and then placed in a growth room with a 16 h photoperiod. n=20. Mean values represented by the same alphabetical letters within each column are not significantly different (Chi-squared and Scheffé's multiple range test, $p < 0.05$).

Medium	Time in dark	% Shoot survival	Shoot length (mm)	% explants producing callus
1	24 h	75 [a]	2.7 [a]	0 [a]
	3 d	85 [a]	3.7 [a]	0 [a]
	7 d	75 [a]	3.8 [a]	0 [a]
	14 d	65 [a]	3.5 [a]	30 [b]
	28 d	80 [a]	3.6 [a]	40 [b]
2	24 h	85 [a]	4.2 [a]	0 [a]
	3 d	95 [a]	3.8 [a]	0 [a]
	7 d	80 [a]	4.1 [a]	0 [a]
	14 d	85 [a]	4.3 [a]	0 [a]
	28 d	75 [a]	6.3 [b]	30 [b]

After the initial experiments, it was considered that the addition of growth regulators to the culture medium might be advantageous. Therefore, a new batch of axillary shoots which were not etiolated, were placed on both media types as shown in Table 2.11, but with 0.1, 0.5 and 1.0 mg l^{-1} BAP incorporated into the culture media (Table 2.12).

A study done on *Pinus patula* showed that adventitious buds formed on embryo explants would inevitably turn brown and die if excised (McKellar, 1993). Only buds attached to the original explant tissue survived and elongated. Although the present experiments used neither embryonic axes nor adventitious buds, it was considered that the same principle might apply. Therefore, in another attempt to obtain elongation, axillary shoots were excised with a piece of stem (1 mm) attached at the base, before being subjected to the different media containing BAP. Unfortunately, the effect of the

1 mm piece of stem attached to the explant without the incorporation of BAP was not tested.

The results shown in Table 2.12 reveal a significant effect of BAP addition on the percentage of axillary shoots with a 1 mm segment of the parent stem, surviving. The inclusion of 0.5 mg l^{-1} BAP to half-strength DKW salts (Medium 2) showed, 100% shoot survival, while the inclusion of 0.1 mg l^{-1} BAP to WPM (Medium 1) effected the lowest shoot survival, which was nevertheless 75% (Table 2.12). The inclusion of BAP in the culture medium resulted in multiplication of the shoots; axillary shoots placed on WPM (Medium 1) with 0.5 mg l^{-1} BAP produced an average of 3.4 shoots per explant, which was significantly higher than the number of shoots produced on half-strength DKW salts, and similar to the number produced when 1.0 mg l^{-1} BAP was included in WPM. Axillary shoot length was significantly affected: shoots on half-strength DKW medium containing 0.1 mg l^{-1} BAP reached 6.2 mm after 12 weeks in culture. However, virtually all explants placed on half-strength DKW medium with the incorporation of BAP formed callus, which may have been responsible for the slower growth rate achieved (Table 2.12).

In many of the treatments tested, axillary shoot growth was very slow or did not occur, and longer axillary shoots as starting material grew more rapidly than shorter ones (results not shown); however, these longer explants would be too large to be dried successfully or to be cooled rapidly – both of which are essential factors for survival of cryopreservation (Pammenter *et al.*, 2002; Baek *et al.*, 2003). The slow growth rate as well as the lack of success in achieving adequate shoot elongation did not encourage the use of axillary shoots as explants for cryopreservation. Therefore, the use of axillary shoots from greenhouse-grown nodal segments as explants for cryopreservation was not considered further in this study. The use of *in vitro* nodal segments, which were considerably smaller, was therefore investigated (section 2.2.2).

Table 2.12: The effect of leaving 1 mm of stem attached to the base of the axillary shoot, on elongation with the incorporation of BAP to the culture media. Medium 1 and 2: WPM and half-strength DKW salts, respectively. n=14-17. Mean values represented by the same alphabetical letters within each column are not significantly different (Chi-squared and Scheffé's multiple range test, $p < 0.05$).

Medium	[BAP] (mg l^{-1})	% shoot survival	Avg. no. of shoots/explant	Shoot length (mm)	% explants producing callus
	0.1	75 [a]	1.2 [a]	5.5 [a]	6 [a]
1	0.5	76 [a]	3.4 [b]	5.4 [a]	47 [b]
	1.0	92 [ab]	2.1 [ab]	4.6 [a]	42 [b]
	0.1	92 [ab]	1 [a]	6.2 [a]	100 [c]
2	0.5	100 [b]	1.6 [a]	4.3 [a]	100 [c]
	1.0	88 [ab]	1.9 [a]	4.0 [a]	94 [c]

2.2.3 Establishment of *in vitro* nodal segments

2.2.3.1 Establishment of *in vitro* plantlets

In vitro nodal segments were obtained from shoots generated by excised zygotic axes cultured under *in vitro* conditions. The establishment of plantlets from excised embryonic axes *in vitro* requires a nutrient supply since, upon excision, the axis is removed from its natural surrounding, where the cotyledons or endosperm, depending on the seed type, provides nutrients for germination. The appropriate nutrient requirements for germination tend to be species specific (Dumet *et al*., 1997). A complication that commonly arises when culturing embryonic axes is the lack of shoot formation, which is suggested to be caused by injury to the shoot apex upon cotyledon excision (Goveia *et al*., 2004; Perán *et al*., 2006). However, in the case of cocoa, axes germinated successfully on various basal media compositions (Table 2.13). Germination was considered successful when embryos formed roots and shoots. Choice of the best basal medium, since all media types investigated facilitated high germination percentages, was made after measuring the growth rate of the *in vitro* seedlings. It was found that MS (Murashige and Skoog, 1962) medium supplemented with 30 g l^{-1} sucrose and 8.0 g l^{-1} agar (Table 2.13), produced seedlings with significantly longer shoots ($p < 0.05$) compared with other basal media tested.

Axis germination *in vitro* can be hampered by the proliferation of fungi or bacteria, which are normally associated with recalcitrant seeds thus necessitating the use of rigorous decontamination protocols (Mycock and Berjak, 1990; Dumet *et al*., 1997; Sutherland *et al*., 2002). Decontamination of cocoa axes with 1% $Ca(OCl)_2$ for 10 min was efficient in the elimination of contaminants associated with the explant, facilitating the establishment of clean cultures in most cases without necessitating further decontamination treatments.

Table 2.13: Effect of different basal media on cocoa embryonic axis germination for establishment of *in vitro* plants**.** MS= Murashige & Skoog (1962), WPM= Lloyd and McCown (1981), DKW= Driver and Kuniyuki (1984). n=20. Mean values represented by the same alphabetical letters within each column are not significantly different (Chi-squared and Scheffé's multiple range test, $p < 0.05$).

Basal salt	% Germination	Root length (mm)	Shoot length (mm)
MS	100 [a]	19.6 [bc]	2.1[a]
½ MS	100 [a]	17.7 [abc]	1.4 [b]
¼ MS	100 [a]	14.9 [a]	1.3 [b]
WPM	90 [a]	16.6 [ab]	1.2 [b]
½ WPM	100 [a]	20.8 [c]	1.5 [b]
DKW	100 [a]	16.6 [ab]	1.4 [b]
½ DKW	85 [a]	17.2 [abc]	1.3 [b]

2.2.3.2 Multiplication of shoots via embryonic axes

Zygotic axes, after 8 - 12 weeks in culture, produced one shoot each, and, at that stage, shoots had two or three nodes, which collectively, afforded too few nodal explants for cryopreservation studies. In addition, cocoa fruits are not locally available, posing further constraints to the number of axes that could be germinated. Multiplication of shoots from embryonic axes was therefore necessary in order to increase the number of nodal segments available. Two strategies were employed: (i) zygotic axes were placed on solidified germination medium (MS medium) containing one of two cytokinins, BAP or 2-iP (Table 2.14), or (ii) zygotic axes were placed in a RITA system with liquid media containing the same concentrations of growth regulators, with three different flushing intervals (Table 2.15). The two cytokinins that were selected are regularly incorporated into culture media to achieve shoot multiplication (Dhooghe and Labeke, 2007).

Use of the solid media showed that increasingly high concentrations of BAP and 2-iP (10 mg l^{-1}) significantly decreased the percentage of zygotic axes producing shoots ($p <$ 0.05, Table 2.14). Even at the lowest concentration of BAP and 2-iP, although all axes produced shoots, the incorporation of the growth regulators into the solid medium did not significantly affect shoot multiplication. Axes germinated on media supplemented with 0.1 and 5.0 mg l^{-1} BAP produced an average of 1.11 and 1.33 shoots respectively, which was not significantly different from shoot production on media containing the other concentrations of the growth regulators.

Shoot length was affected by the incorporation of growth regulators into the culture media. High concentrations of growth regulators negatively affected the growth rate, with axes germinated in the presence of 10 mg l^{-1} of either BAP or 2-iP showing the shortest shoots, 2.5 and 2.1 mm respectively. The incorporation of 0.1 mg l^{-1} 2-iP into the culture medium significantly increased shoot length compared with the other treatments, as shown in Table 2.14 (and cf Table 2.13). Very low contamination levels were recorded with no significant difference in contamination frequency amongst treatments. However, the use of solid medium with the incorporation of either of the cytokinins did not promote shoot multiplication; therefore, the use of liquid medium was investigated.

Table 2.14: Effect of BAP and 2-iP on shoot multiplication via cocoa embryonic axes using solid medium. The solid medium included MS nutrients, 30 g l^{-1} sucrose and 8.0 g l^{-1} agar. BAP= Benzyl amino purine, 2-iP= 2-isopentenylamino purine. n=20. Mean values represented by the same alphabetical letters within each column are not significantly different (Chi-squared and Scheffé's multiple range test, $p < 0.05$).

Growth Regulator (mg l^{-1})		% explants producing shoots	Avg. no. of shoots produced /explant	Shoot length (mm)	% Contamination
BAP	2-iP				
0.1		100 [a]	1.11 [a]	7.7 [a]	0 [a]
1.0		90 [ab]	1.00 [a]	4.7 [a]	5 [a]
5.0		75 [b]	1.33 [a]	3.4 [a]	5 [a]
10		70 [b]	1.00 [a]	2.5 [a]	0 [a]
	0.1	100 [a]	1.00 [a]	17.6 [b]	0 [a]
	1.0	80 [ab]	1.00 [a]	5.4 [a]	10 [a]
	5.0	80 [ab]	1.00 [a]	7.4 [a]	0 [a]
	10	60 [b]	1.00 [a]	2.1 [a]	5 [a]

The strategy was the use of the RITA system to pulse the axes with the liquid medium containing the cytokinins at different concentrations for varying time intervals. The RITA system is a powerful aid for the mass propagation of plant material, and has been used in the large-scale production of a variety of species for commercial purposes (Afreen *et al*., 2002; McAlister *et al*., 2005). The benefit of the RITA system is that it supplies readily available nutrient efficiently, as well as providing adequate aeration, preventing the possibility of an anoxic environment, and hence promoting tissue growth (He *et al*., 2007). In this study, shoot multiplication was achieved via the RITA system, and provision of 1.0 mg l^{-1} of either BAP or 2-iP resulted in more that two shoots per explant (Figure 2.4). Shoot multiplication was best using the 30 sec flush/30 min rest regime (Table 2.15). There were no distinct differences between the effects of the two

growth regulators in terms of shoot multiplication. Shoot length was also significantly influenced by the flushing intervals, the frequent flushing regime, 30 sec flush/30 min rest, producing generally longer shoots compared with the other two flushing intervals.

The major problem with the system, though, was high contamination level. This concurs with other studies, where contamination is carried through the system in the liquid medium (McAlister *et al*., 2005; Snyman *et al*., 2007). Due to the limitation of cocoa seeds, this experiment could not been repeated with the implementation of stricter decontamination strategies, or the addition of plant preservative medium (PPM) which has been reported to reduce contamination of sugarcane somatic embryos when using the RITA system (Snyman *et al*., 2007). The shoots that did multiply, as well as embryonic axes that germinated from previous batches of cocoa seeds received, were used for subsequent experiments.

Figure 2.4: Shoot multiplication obtained from embryonic axes flushed for 30 sec every 30 min with liquid medium containing 1.0 mg l^{-1} BAP. Scale bar = 2 mm

Table 2.15: Effect of BAP and 2-iP on shoot production by cocoa embryonic axes using the RITA system with three different flush intervals, for 3 days. n=20. Mean values represented by the same alphabetical letters within columns are not significantly different (Chi-squared and Scheffé's multiple range test, $p < 0.05$).

Flush interval	Growth Regulator ($mg\ l^{-1}$)		% explants producing shoots	Avg. no. of shoots produced/ explant	Shoot length (mm)	% Contamin-ation
	BAP	2-iP				
30 sec flush, 30 min rest	0.1		55 ^{bc}	1.4 ^{ab}	25.6 ^{a}	45 ^{b}
	1.0		100 ^{a}	2.3 ^{b}	12.9 ^{ab}	0 ^{a}
	5.0		60 ^{b}	1.5 ^{ab}	11.7 ^{ab}	30 ^{b}
		0.1	70 ^{b}	1.4 ^{ab}	8.6 ^{b}	30 ^{b}
		1.0	50 ^{bc}	1.9 ^{ab}	18.9 ^{ab}	50 ^{b}
		5.0	35 ^{cd}	1.4 ^{ab}	15.9 ^{ab}	30 ^{b}
1 min flush, 1 h rest	0.1		100 ^{a}	1.1 ^{a}	25.8 ^{a}	0 ^{a}
	1.0		-	-	-	100 ^{c}
	5.0		80 ^{a}	1.4 ^{ab}	5.1 ^{b}	20 ^{b}
		0.1	100 ^{a}	1.2 ^{a}	11.7 ^{ab}	0 ^{a}
		1.0	100 ^{a}	1.1 ^{a}	4.9 ^{b}	0 ^{a}
		5.0	30 ^{cd}	1.0 ^{a}	4.8 ^{b}	20 ^{b}
1 min flush, 3 h rest	0.1		50 ^{bc}	1.0 ^{a}	6.9 ^{b}	50 ^{b}
	1.0		10 ^{d}	1.0 ^{a}	5.5 ^{b}	90 ^{c}
	5.0		80 ^{ab}	1.0 ^{a}	6.6 ^{b}	20 ^{b}
		0.1	55 ^{bc}	1.0 ^{a}	4.8 ^{b}	45 ^{b}
		1.0	-	-	-	100 ^{c}
		5.0	50 ^{bc}	1.0 ^{a}	4.9 ^{b}	30 ^{b}

2.2.3.3 Axillary bud culture via *in vitro* nodal segments

Three different treatments were used to achieve bud break from *in vitro* nodal segments. However, prior to the bud-break step, explants had to be pre-treated (as described for axillary buds in section 2.2) to prevent the lethal effects of phenolics (observed with explants from greenhouse-grown plants) exuded into the culture medium. *In vitro* nodal segments were thus soaked for 10 min in 0.5% ascorbic acid followed by 5 min decontamination with 1% $Ca(OCl)_2$ even though explants were from seedlings produced in a sterile environment. Two established protocols for cocoa (Flynn *et al*., 1990; Traore *et al*., 2003) and the protocol established in this study for nodal segments derived from greenhouse-grown plants were used to determine the best bud-break medium. There was no significant difference in the percentage of *in vitro* nodal segments producing buds or the number of buds produced per explant among the protocols (Table 2.16). However, the protocol established by Traore *et al*. (2003) was taken to be the best since this treatment resulted in the highest percentage bud-break, 70%.

Table 2.16: Bud-break of *in vitro* nodal segments using three different media compositions. n=20. Mean values represented by the same alphabetical letters within each column are not significantly different (Chi-squared test, $p < 0.05$).

Treatment	% explants producing buds	Avg. no. of buds/explant	Time taken for bud-break (days)
Flynn *et al*. (1990)	40 [a]	1 [a]	31
Traore *et al*. (2003)	70 [a]	1 [a]	28
WPM	50 [a]	1 [a]	35

Nodal segments from *in vitro* seedlings and those from greenhouse-grown plants differed with respect to the percentage of explants producing buds and the rate of growth of the axillary shoot; the former required between 28 to 35 days for bud-break to occur compared with the latter, which occurred after 17 days.

In an attempt to reduce the time required for bud-break of *in vitro* seedling-derived nodal segments, three concentrations of BAP were included into the culture media (Table 2.17). The inclusion of BAP reduced the time for bud-break to 24 days and

enhanced shoot multiplication (cf Table 2.16). The addition of 0.5 mg l^{-1} BAP to the culture medium resulted in an average of two shoots per nodal segment. In the study performed by Traore *et al*. (2003) cocoa nodal and apical explants were exposed to a medium containing a (synthetic) cytokinin, thidiazuron (TDZ), but this did not improve the extent of multiplication of shoots, nor the time taken for shoot formation; rather there was increased formation of callus at the base of the explants. In this study, callus formation was also evident on explants exposed to BAP (results not shown).

Buds that had broken, but were still attached to the nodal segment, were transferred to a medium devoid of growth regulators for elongation. On reaching a length of 10 - 15 mm, 50% of shoots rooted as (described in 2.2.3 for axillary shoots derived from greenhouse nodal segments; details not shown).

Table 2.17: Axillary bud-break via nodal segments from *in vitro* established plantlets on half strength DKW salts including BAP. n=20. Mean values represented by the same alphabetical letters within each column are not significantly different (Chi-squared test and Scheffé's multiple range test, $p < 0.05$).

Medium	[BAP] (mg l^{-1})	% explants producing buds	Avg. no. of buds/explant	Time taken for bud-break (days)
Traore *et al*. (2003)	0.1	65 [a]	1.5 [a]	24
	0.5	80 [a]	2.0 [b]	24
	1.0	75 [a]	1.7 [ab]	24

2.2.4 *In vitro* nodal segments as explants for cryopreservation

2.2.4.1 Optimisation of explant size

Cryopreservation necessitates the use of small explants since, in general, the smaller and less developmentally complex the specimen the more easily can the explant be successfully cryopreserved (Berjak *et al*., 1996; Wesley-Smith *et al*., 1998). Nodal segments were excised and categorised into three classes i.e. 2 - 3 mm, 4 - 5 mm and 6 - 7 mm to obtain an appropriate explant size. Bud-break occurred in a significantly lower

($p < 0.05$) percentage of 2 - 3 mm explants compared with explants from the 6 - 7 mm class (Table 2.18).

There was no significant difference in the number of buds per explant across explant sizes. The time required for bud-break was longer for the shorter explants compared with 28 days for 6 - 7 mm long explants. The smallest explant size that could be used for subsequent dehydration and cooling experiments was taken to be 6 - 7 mm since these responded the best in culture. Nevertheless, the size of these explants potentially mitigated against successful cryopreservation.

Table 2.18: Effect of explant size on bud-break to establish explant size for cryopreservation. n=20. Mean values represented by the same alphabetical letters within each column are not significantly different (Chi-squared test, $p < 0.05$).

Medium	Explant size (mm)	% Bud-break	Avg. no. of buds/explant	Time taken for bud-break (days)
Traore *et al*. (2003)	2 - 3	20 [a]	1 [a]	35
	4 - 5	40 [ab]	1 [a]	31
	6 - 7	65 [b]	1 [a]	28

2.2.4.2 Optimisation of culture vessel

In an attempt to improve on the 65% bud-break achieved using the 6 – 7 mm explants, in 65-mm Petri dishes, the effect of different culture vessels were tested. The volume of the air space in a culture vessel has been suggested to impinge on explant growth for a variety of reasons (e.g. George, 1996; Hartmann *et al*., 2001). In this study, bud-break achieved in three different types of culture vessel i.e. 65-mm and 90-mm Petri dishes and 25 mm x 100 mm glass culture tubes, was assessed. The percentage of explants showing bud-break was not significantly affected by the type/volume of the culture vessel ($p > 0.05$, Table 2.19) but use of culture tubes facilitated the highest percentage bud-break, 80%, so tubes were selected for all subsequent experiments. In addition, the time taken for bud-break to occur for explants in the culture tubes was 24 days, compared with 31 days and 28 days for 65-mm and 90-mm Petri dishes, respectively.

Culture tubes or larger culture vessels have been shown to be better culture vessels compared with Petri dishes for e.g. rice callus cultures (Adkins *et al*., 1993). Those authors showed reduced growth and necrosis of several rice (*Oryza sativa*) cultivars when explants were cultured in Petri dishes rather than tubes. It is suggested that larger culture vessels compared with Petri dishes, allow for the dilution/dispersion of ethylene produced by explants, which is known to have detrimental effects on the tissue upon accumulation in the culture vessel (George, 1996; Adkins *et al*., 1993). Larger culture vessels also provide more oxygen and a lesser concentration of CO_2 in the headspace, between successive sub-culturing.

Table 2.19: The effect of different culture vessels on bud break frequency. n=15. Mean values represented by the same alphabetical letters within each column are not significantly different (Chi-squared and Scheffé's multiple range test, $p < 0.05$).

Culture vessel	% Bud-break	Avg. no. of buds developing/explant	Time taken for bud-break (days)
65-mm Petri dish	53 [a]	1 [a]	31
90-mm Petri dish	66 [a]	1 [a]	28
Culture tube	80 [a]	1.5 [a]	24

2.2.4.3 Dehydration of nodal segments

Plants cells that are cultured consist of approximately 95% water facilitating ice formation upon exposure to sub-zero temperatures (Reinhoud *et al*., 2000). Thus, cells have to be dehydrated artificially to protect them from lethal ice damage, which, in most cases, is responsible for tissue death during cryopreservation (e.g. Burke *et al*., 1976; Sakai *et al*., 1990). The reduction of the amount of intracellular water, however, results in concentration of solutes and potentially, plasmolysis of cells, and both events could also lead to cell death (Reinhoud *et al*., 2000).

The original water content of cocoa nodal segments in this study was between 3.8 and 4.8 g g^{-1} dry mass, which was considerably lower than that reported for yam, 12.18 g g^{-1} (Quain *et al*., 2007) but higher than the 2.04 g g^{-1} reported for citrus (Santos and Stushnoff, 2003), 1.74 g g^{-1} for *Quercus* species (González-Benito, 2002) and 1.42 g g^{-1}

for wasabi (Potts and Lumpkin, 1997). In all cases, however, a dehydration step would be necessary to lower the water content of explants before attempting cooling in liquid nitrogen. Tissues can be dehydrated using one or more of a variety of strategies (see Chapter 1) but in this study, explants were dehydrated via silica gel, flash drying and by using an osmoticum.

Silica gel, a drying agent, has been used in several cryopreservation studies to dehydrate different types of explants, including embryonic axes (Fu *et al*., 1993); shoot apices (Malaurie *et al*., 1998); and somatic embryos (Mycock *et al*., 1995). Presently, the cocoa explants were initially dried at intervals of 1 h for a total of 6 h. However, after the first hour, no explants survived after being placed on filter paper over activated silica gel and dehydrated to a water content of 0.78±0.29 g g^{-1} (Table 2.20). Furthermore, in these trials dehydration of explants for longer than 2 h resulted in no substantial further water loss, the water content remaining at ±0.23 g g^{-1}, when all remaining water in the tissue could be referred to as structure bound (unfreezable) water (Volk and Walters, 2006). No further studies were carried out using shorter dehydration periods over silica gel due to limitation of material but mainly because even at a mean tissue water content of 0.78 g g^{-1}, survival of these large explants upon liquid nitrogen exposure, was considered to be highly unlikely (e.g. Wesley-Smith *et al*., 2001).

Table 2.20: Desiccation of *in vitro* nodal segments using silica gel. Explants were placed in 100 ml culture bottles on a piece of sterilised filter paper over approximately 50 g of activated silica gel. Following each dehydration period, water content (n=5) and viability (n=15) was assessed. Mean values represented by the same alphabetical letters are not significantly different (Chi-squared test, $p < 0.05$).

Slow drying duration	Water content ($g\ g^{-1}$)	% Bud-break	Avg. no. of buds developing/ explant	Time taken for bud-break (days)
0	3.79 ± 0.24	70 ^{a}	1	28
1	0.78 ± 0.29	0 ^{b}	-	-
2	0.23 ± 0.07	0 ^{b}	-	-
3	0.21 ± 0.12	0 ^{b}	-	-
4	0.23 ± 0.06	0 ^{b}	-	-
5	0.07 ± 0.01	0 ^{b}	-	-
6	0.23 ± 0.09	0 ^{b}	-	-

The next attempt to dehydrate nodal segments was performed using a flash dryer. The flash drying apparatus is designed to dehydrate small explants rapidly by the circulation of dry air over them (Berjak *et al*., 1989; Pammenter *et al*., 2002). Rapid dehydration is suggested to curtail metabolism-linked damage that occurs if the cells pass slowly through "intermediate" water contents (Pammenter *et al*., 1998, 2002). No nodal segments flash-dried for longer than 20 min survived and, in fact, after this drying time when mean water content was 1.13 $g\ g^{-1}$, only 10% of explants survived (Table 2.21).

In this experiment, nodal segments flash dried for 80 min had higher water contents than nodal segments dried for 60 min (Table 2.21), which could be due to variability in the original water contents but (what is more likely) was probably a function of the small sample size (n=5) used for water content determinations. Neither silica gel dehydration nor flash drying achieved sufficiently low water contents, whilst maintaining viability, for explant cryopreservation. Therefore, an alternative dehydration strategy was investigated, entailing the use of an osmoticum.

Table 2.21: Desiccation of *in vitro* nodal segments using a flash drier. Following each flash drying duration water content (n=5) and viability (n=15) was assessed. Mean values represented by the same alphabetical letters are not significantly different (Chi-squared test, $p < 0.05$).

Flash drying duration	Water content ± SD (g g^{-1})	% Bud-break	Avg. no. of buds developing/ explant	Time taken for bud-break (days)
0	3.78 ± 0.24	70 [a]	1 [a]	28
10	3.45 ± 1.23	60 [a]	1 [a]	31
20	1.13 ± 0.54	10 [b]	1 [a]	31
40	0.53 ± 0.22	0	-	-
60	0.26 ± 0.12	0	-	-
80	0.50 ± 0.18	0	-	-

The use of sucrose-enriched medium to desiccate vegetative explants is relatively common (e.g. Zhao *et al*., 2001), and sucrose also exhibits a cryoprotectant effect (Panis *et al*., 2002). It has been suggested that exposing explants to increasing concentrations of sucrose for set durations, will reduce the osmotic shock consequent upon their being placed directly on high medium containing concentrations of sucrose (Seijo, 2000; Moges *et al*., 2004; Reed *et al*., 2005). In this study, cocoa nodal segments tolerated 24 h on medium containing 0.09 M sucrose, where 70% survival was obtained, but when the explants were subsequently transferred to 0.3 M sucrose containing medium for a further 24 h, there was a decline to 55% of nodal segments surviving (Table 2.22). Nodal segments were shown to be very sensitive to sucrose concentrations above 0.3 M, none surviving on media with 0.5 M sucrose or higher (Table 2.22).

None of the methods used to this point to dehydrate cocoa nodal segments to water contents that would facilitate successful cooling, were successful.

Table 2.22: Desiccation of *in vitro* nodal segments via sequential sucrose dehydration. Nodal segments were incubated on media containing increasing concentrations of sucrose for 24 h periods at each respective sucrose concentration. Following incubation at each sucrose concentration, water content (n=5) and viability (n=15) were assessed. Mean values represented by the same alphabetical letters within each column are not significantly different (Chi-squared and Scheffé's multiple range test, $p < 0.05$).

Sucrose Concentration	Water Content ± SD ($g\ g^{-1}$)	% Bud-break	Avg. no. of buds developing/explant	Time taken for bud-break (days)
0.09 M	5.86± 1.27	70 [a]	1 [a]	21
0.3 M	4.07± 0.20	55 [a]	1 [a]	28
0.5 M	3.21± 0.39	0 [b]	-	-
0.75 M	2.25± 0.13	0 [b]	-	-
1 M	1.68± 0.20	0 [b]	-	-

2.2.4.4 Sucrose pre-culture

Sucrose pre-culture involves the incubation of explants on only one concentration of sucrose for a set duration as opposed to sucrose dehydration that requires the movement of explants from a low concentration of sucrose to sequential higher concentrations. Pre-culturing of explants on sucrose medium has been found to be beneficial in inducing dehydration and freeze tolerance (Quain *et al*., 2007). Studies carried out on tropical crops have mostly used sucrose at concentrations from 0.09 M to 1 M for two to three days before attempting cooling (Takagi, 2000). In this study, the culturing of cocoa *in vitro* nodal segments on 0.09 M, 0.3 M, 0.5 M, 0.75 M or 1 M sucrose for 24 h or 3 days had a significant negative effect on the percentage of explants producing buds. None of the explants survived on 1 M sucrose after either time interval. The best pre-treatment was pre-culture on medium incorporating 0.3 M sucrose for a 24 h period, after which 50% of explants produced buds and water content declined to a mean of 3.88 $g\ g^{-1}$ (Table 2.23). This treatment has also been used for cassava shoot tips (Charoensub *et al*., 1999), shoot tips of *Ipomoea batata* (Pennycooke and Towill, 2001) and several species of *Dioscorea* (Leunufna and Keller, 2005). However, in the present

experiments, the mean water content, at 3.88 g g^{-1}, was too high for the cocoa explants to survive cryogen exposure (Quain *et al*., 2007).

Exposure of explants without the pre-culture step to concentrations of sucrose higher than 0.3 M for 24 h incubation periods was lethal (Table 2.22). In contrast, pre-culturing of explants for 24 h on 0.3, 0.5, and 0.75 M sucrose facilitated explant survival (Table 2.23). However, after the longer exposure period (3 d) to 0.3 and 0.5 M sucrose poorer survival was obtained. These results indicate detrimental effects of both sucrose concentration and duration of exposure. Furthermore, water contents, although substantially lowered after culture on sucrose-containing media, remained at levels that would potentially obviate survival of cryopreservation.

Table 2.23: The effect of two pre-treatment incubation periods on different concentrations of sucrose. Nodal segments were placed on medium incorporating the respective sucrose concentrations for either 24 h or 3 d. Following each sucrose pre-treatment, water content (n=5) and viability (n=15) were assessed. Mean values represented by the same alphabetical letters are not significantly different (Chi-squared test, $p < 0.05$).

Precult-ure period	Sucrose Concent-ration (M)	Water Content ± SD ($g\ g^{-1}$)	% Bud-break	Avg. no. of buds developing/ explant	Time taken for bud-break (days)
24 h pre-culture	0.09 (Control)	4.50 ± 0.49	60 [c]	1 [a]	28
	0.3	3.88 ± 0.64	50 [bc]	1 [a]	35
	0.5	2.35 ± 0.87	30 [bc]	1 [a]	35
	0.75	2.29 ± 0.32	20 [b]	1 [a]	35
	1.0	2.19 ± 0.09	0 [a]	-	-
3 d pre-culture	0.09 (Control)	4.64 ± 0.55	60 [c]	1 [a]	28
	0.3	2.55 ± 0.11	20 [b]	1 [a]	35
	0.5	2.45 ± 0.52	20 [b]	1 [a]	35
	0.75	1.90 ± 0.58	20 [b]	1 [a]	35
	1.0	1.71 ± 0.11	0 [a]	-	-

2.2.4.5 Vitrification and exposure to -196˚C (liquid nitrogen)

The vitrification technique has been successfully used to cryopreserve different types of explants from species of both temperate and tropical provenance (Table 1.1). This procedure relies on the transition of an aqueous solution directly from the liquid phase to an amorphous, glassy phase, avoiding crystalline ice formation (Fahy *et al*., 1984; Engelmann, 1997). However, recent studies performed by Volk and Walters (2006) suggest that the protective mechanism of PVS2 is based on a partial restriction of molecular mobility and/or a disorganization of ice crystal structure rather than the prevention of ice crystallization by forming a glass. Those authors outline mechanisms

that explain how PVS2 aids cryoprotection of shoot tips by replacing cellular water and changing freezing behaviour of water remaining in cells.

In this study, the vitrification technique was used since explants could not be adequately dehydrated while retaining viability, using the various methods described above. Cocoa nodal segments were first pre-cultured on 0.3 M sucrose and then treated with a loading solution prior to exposure to the PVS2 solution. Pre-culturing of explants on sucrose-enriched medium for varying periods has contributed to success when followed by vitrification (Keller, 2005; Sant *et al*., 2006; Quain *et al*., 2007). According to Sakai (2000) to induce dehydration tolerance, excised shoot-tips from *in vitro* grown plantlets are pre-cultured on media with high sucrose concentrations (0.3 – 0.6 M) for 16 h and then treated with a mixture of 2 M glycerol plus 0.4 M sucrose, the loading solution, for 20 min before dehydration with PVS2. The loading solution used in the current study, which contained half strength DKW salts, 2 M glycerol and 0.4 M sucrose, is generally thought to have the effect of reducing the sensitivity of the tissue to the highly concentrated vitrification solutions (Engelmann, 1997; Sakai, 2000). In this study, 30% of the cocoa nodal segments exposed to the loading solution for 20 min survived, and required 31 days for bud-break to occur (Table 2.24). The nodal segments survived exposure to PVS2 solution only for durations of 10 and 15 min, after which there was no significant difference in percentage bud-break between explants dehydrated at 0˚C or 25˚C (Table 2.24), although, according to Engelmann (1997), performing the dehydration step at 0˚C instead of room temperature reduces the toxicity of vitrification solutions thus expanding the period of exposure durations facilitating explant survival. In the current investigations, after vitrification, cryotubes containing cocoa explants (without PVS2) were plunged into liquid nitrogen. However, no explants survived after exposure to liquid nitrogen, rapid thawing for 2 min at 40˚C and unloading in half strength DKW salts and 1.2 M sucrose. The unloading step aims at removing progressively the vitrification solution in order to reduce osmotic shock (Engelmann, 1997). The limitations associated with this technique are chemical phytoxicity and osmotic stress damage from PVS2 solution (Sant *et al*., 2006), which could have been the cause of tissue mortality in this study, even though treatment durations were

relatively short. Once again, the unfortunate limitation of stock material prevented refinement of the vitrification procedures for the cocoa explants.

Table 2.24: The effect of cooling *in vitro* nodal segments using the vitrification cryopreservation technique. Following the PVS 2 treatment, water content (n=5) and viability (n=15) was assessed and post freezing viability (n=15) was assessed. Mean values represented by the same alphabetical letters are not significantly different (Chi-squared test, $p < 0.05$).

Treatment and duration	Water Content ± SD (g g^{-1})	(%) bud break				Time taken for bud break (days)			
		0˚C		25˚C		0˚C		25˚C	
		-LN	+LN	-LN	+LN	-LN	+LN	-LN	+LN
control	4.80± 0.34	NT*		70 [c]	NT	NT		24	NT
0.3 M Sucrose 24 h	4.38± 0.32			55 [bc]				28	
LS 20 min	3.12± 0.27			30 [ab]				31	
PVS 2 (min)									
10	3.04± 0.13	20 [a]	0	10 [a]	0	28	-	28	-
15	3.02± 0.43	10 [a]	0	10 [a]	0	28	-	28	-
20	2.51± 0.31	0	0	0	0	-	-	-	-
25	2.66± 0.07	0	0	0	0	-	-	-	-
30	2.67± 0.22	0	0	0	0	-	-	-	-

*NT – Not tested

2.2.4.6 Two-stage cooling

Two-stage cooling is a classical technique based on freeze-induced dehydration where ice-formation occurs extracellularly, intracellular water being drawn outwards to centres of ice nucleation (Kartha, 1985). The explants are usually treated with cryoprotectants, which play the role of maintaining tissue integrity and/or increasing the osmotic potential of the external medium (Krishnapillay, 2000). The osmotic gradient set up

promotes movement of water out of the cells, with consequent intracellular dehydration. In this study, nodal segments were treated with two cryoprotectant solutions prior to slow cooling, sucrose in combination with glycerol, and glycerol alone, since several studies have revealed that these cryoprotectant solutions promote survival of freezing and thawing (e.g. Matsumoto and Sakai, 1995; Sershen *et al.*, 2007). The effect of pre-culturing nodal segments and then cryoprotecting them, was also assessed (Treatments c and d).

Cryoprotection of cocoa nodal segments prior to slow cooling was associated with very low survival percentages, glycerol-cryoprotected explants (Treatment e), however, survived slightly better than explants treated with a combination of sucrose and glycerol (Treatment f) (Table 2.25). Pre-culturing nodal segments on 0.3 M sucrose prior to cryoprotection with glycerol (Treatment c) was associated with increased bud-break, compared with no pre-culture (Treatment e). There was, however, no explant survival post thawing, following slow cooling to -40˚C (using Mr Frosty®) and then plunging cryovials into liquid nitrogen (Tables 2.26 and 2.27). As explants were thawed rapidly in a 40˚C water bath for 2 min to reduce the risk of ice recrystallisation during the thawing process (Krishnapillay, 2000), it seems probable that freezing itself was lethal.

Table 2.25: The effect of different pre-treatments on *in vitro* nodal segments prior to slow cooling. Following each pre-treatment, water content (n=5) and viability (n=15) was assessed. Mean values represented by the same alphabetical letters are not significantly different (Chi-squared test, $p < 0.05$).

Treatment	Water content ($g\ g^{-1}$)	% Bud break	Time taken for bud-break (days)
a	3.83 ± 0.30	60 [c]	28
b	3.23 ± 0.40	40 [bc]	31
c	4.77 ± 1.13	20 [b]	31
d	3.97 ± 0.52	10 [ab]	31
e	3.74 ± 1.52	10 [ab]	31
f	2.34 ± 0.47	0 [a]	-

a - no treatment (control); **b** - pre-culture (0.3 M sucrose for 24 h); **c** - pre-culture (0.3 M sucrose for 24 h) followed by cryoprotection (5% glycerol 15 min, 10% glycerol 15 min); **d** - pre-culture (0.3 M sucrose for 24 h) followed by cryoprotection (5% sucrose + glycerol 15 min, 10% sucrose + glycerol 15 min); **e** - cryoprotection (5% glycerol 15 min, 10% glycerol 15 min); **f** - cryoprotection (5% sucrose + glycerol 15 min, 10% sucrose + glycerol 15 min).

2.2.4.7 Rehydration

Non-injurious rehydration of explants after exposure to liquid nitrogen is also vital for survival. Explants were rehydrated using various strategies: for explants that were not cryoprotected the effect of not decontaminating and not rehydrating explants was tested against decontamination only, and decontamination in combination with 15 min rehydration in CaMg solution. None of the explants survived after freezing, thawing and rehydrating. However, it was evident that a decontamination step was necessary since most of the explants that were not treated for 5 min in 1% CaOCl were contaminated with bacteria (Table 2.26).

Table 2.26: The effect of different rehydration techniques after retrieval from liquid nitrogen. Following rehydration, viability (n=15) was assessed. Mean values represented by the same alphabetical letters are not significantly different (Chi-squared test, $p < 0.05$).

Treatment	No rehydration No decontamination		No rehydration decontamination		Rehydration decontamination	
	% Bud-break	% Contamination	% Bud-break	% Contamination	% Bud-break	% Contamination
a	0 [a]	80 [a]	0 [a]	10 [a]	0 [a]	20 [a]
b	0 [a]	90 [a]	0 [a]	10 [a]	0 [a]	10 [a]

a - no treatment (control); **b** - pre-culture (0.3 M sucrose for 24 h)

Explants that were cryoprotected were first unloaded in a 5% solution of the appropriate cryoprotectant solution post-thawing before being either decontaminated, or not, and then rehydrated in CaMg. The removal of the cryoprotectant after retrieval of the plant material from the cryogen is often necessary since the cryoprotective agents could be toxic to plant cells after thawing (Mycock *et al*., 1991; Watanabe, 2000). However, these rehydration strategies presently used had no impact, survival recorded as zero in all cases (Table 2.27).

Table 2.27: The effect of unloading explants for 15 min in a 5% solution of the cryoprotectant used prior to cooling. Following rehydration, viability (n=15) was assessed. Mean values represented by the same alphabetical letters are not significantly different (Chi-squared test, $p < 0.05$).

Treat-ment	No unloading no rehydration decontamination		No unloading rehydration decontamination		Unloading rehydration decontamination	
	% Bud-break	% Contamination	% Bud-break	% Contamination	% Bud-break	% Contamination
c	0^{a}	10^{ab}	0^{a}	0^{a}	0^{a}	20^{a}
d	0^{a}	0^{a}	0^{a}	0^{a}	0^{a}	10^{a}
e	0^{a}	20^{b}	0^{a}	20^{b}	0^{a}	20^{a}
f	0^{a}	10^{ab}	0^{a}	40^{b}	0^{a}	30^{a}

c - pre-culture (0.3 M sucrose for 24 h) followed by cryoprotection (5% glycerol 15 min, 10% glycerol 15 min); **d** - pre-culture (0.3 M sucrose for 24 h) followed by cryoprotection (5% sucrose + glycerol 15 min, 10% sucrose + glycerol 15 min); **e** - cryoprotection (5% glycerol 15 min, 10% glycerol 15 min); **f** - cryoprotection (5% sucrose + glycerol 15 min, 10% sucrose + glycerol 15 min).

Cocoa *in vitro* nodal segments did not respond well to either of the cryopreservation techniques used or to any dehydration pre-treatments applied in this study. It thus appears that the use of nodal segments can be precluded as an explant choice for cryopreservation of cocoa. The major problem so far identified is the inability of these explants to withstand dehydration to water content levels appropriate for cooling (freezing). The establishment of cryopreservation protocols for tropical woody species is considered challenging since intensive investigations in our laboratory, e.g. on *Ekebergia capensis, Cordyla africana* as well as *Trichilia* spp. have yielded negative results with the use of nodal segments (unpublished data). Quain (pers comm[1]) has encountered similar problems with a tropical yam species, as well as with nodal explants of Frafra potato (*Solenostemon rotundifolius*). For all these species, material

[1] M.D. Quain, Tissue Culture Laboratory, Department of Botany, University of Ghana, Legon; Accra, Ghana.

seems to be at very high initial water contents, highly sensitive primarily to desiccation, and to cooling, which might be attributed to their tropical provenance. In addition, the composition of the tissue could be too complex, leading to different responses in the various tissue types, to the necessary manipulations.

Generally, where axillary buds have proved to be good explants for cryostorage, the species are temperate and in most cases the buds are dormant over winter (Towill and Forsline, 1999; Towill *et al*., 2004). This may be correlated with relatively low water contents which would afford resilience to freezing air-temperatures during winter (Towill *et al*., 2004). Certainly, this is not the case for tropical species whether woody or not.

Therefore, the use of less complex explants such as meristems, or clumps of meristematic cells should be investigated. Other possibilities are embryogenic callus or somatic embryos, as described below.

2.2.5 Somatic embryo induction

Somatic embryogenesis is the formation of embryo-like structures from somatic cells through asexual propagation (e.g. Terzi and Loschiavo, 1990; George, 1996). Somatic embryos are commonly used in micropropagation, genetic engineering, synthetic seed formation and for conservation efforts. There has been much work towards development of a somatic embryogenesis protocol for cocoa (listed in Table 1.3, Chapter 1), the established protocol for which utilises inflorescence explants as the primary source of material (Li *et al*., 1998, Tan and Furtek, 2003). However, cryopreservation of cocoa somatic embryos (see Chapter 1) has resulted in only 33% of explants forming plantlets (Fang *et al*., 2003).

Cocoa plants are not indigenous to South Africa, they require tropical conditions for optimal growth, and hence are not locally cultivated. Therefore, inflorescence explants were not available necessitating the use of alternative primary explant material for the induction of somatic embryos in this study. Leaves from stock plants maintained in the greenhouse, and cotyledons available when fruits were air-freighted from Ghana or

Nigeria, were thus used. Explants from leaves (Litz, 1986) and cotyledons (Aguilar *et al*., 1992) have been used in previous studies for the initiation of cocoa somatic embryos.

2.2.5.1 Induction of somatic embryos via leaf explants

The use of leaf explants for the induction of somatic embryos, which can be the basis of clonal propagation, is advantageous, as material is always available from stock plants. Leaf explants used in the present study were taken from seedlings in the relatively sterile greenhouse environment. Explants were successfully decontaminated via the two-step procedure established for nodal segments (see section 2.2.1.1). Treatment of the leaf segments with 1% NaOCl for 10 min prior to trimming followed by immersion in 1% $Ca(OCl)_2$ for 5 min after trimming, eliminated fungal contamination and reduced bacterial contamination significantly ($p < 0.05$, Table 2.28).

Table 2.28: Decontamination of leaf explants for somatic embryo induction. For treatment 5 explants (approximately 40 x 50 mm) were first treated with 1% NaOCl, subsequently trimmed to 10 mm x 10 mm explants and then treated with 1% $Ca(OCl)_2$ with three sterile distilled water rinses between decontaminants. n=50. Mean values represented by the same alphabetical letters are not significantly different (Chi-squared test, $p < 0.05$).

Treatment	Time (min)	(%) Contamination	
		Fungal	Bacterial
1% NaOCl	5	10 [a]	50 [b]
1% NaOCl	10	10 [a]	40 [ab]
1% $Ca(OCl)_2$	5	5 [a]	15 [ab]
1% $Ca(OCl)_2$	10	5 [a]	15 [ab]
1% NaOCl 1% $Ca(OCl)_2$	10 5	0 [a]	10 [a]

The induction of somatic embryogenesis can follow a direct or an indirect route. The direct route generally depends on the type of tissue cultured on the induction medium, as well as on the species (George, 1996). Certain explants have the capacity to form embryos on the appropriate nutrient medium without manipulation of external stimuli including the provision of growth regulators. This is illustrated, for example, by zygotic axes and cotyledon explants of *Protea cynaroides* where somatic embryos formed directly from both explant types upon incubation on MS medium (Wu *et al*., 2007). However, this is not the situation for all species, with wide variation occurring in successful somatic embryogenesis per explant type in response to external stimuli (e.g. George, 1996). The method used in this study for the induction of somatic embryogenesis was via an indirect route, involving formation of embryos via an intervening callus stage.

Different basal media have been used to induce somatic embryogenesis in cocoa (Tan and Furtek, 2003), with the use of MS medium being initially employed (Duhem *et al*., 1989; Chatelet *et al*., 1992). However, in recent studies using inflorescence explants, DKW medium has been utilised (Li *et al*., 1998; Tan and Furtek, 2003), the main difference being higher concentrations of calcium, sulphur and magnesium present in this medium (Saadat and Hennerty, 2002; Tan and Furtek, 2003).

In this study, both MS and DKW medium were used in attempts to induce somatic embryogenesis from both leaf and cotyledons explants. However, somatic embryogenesis was not promoted when MS medium supplemented with various concentrations of 2,4-D or 2,4-D in combination with either BAP or TDZ, was used (Table 2.29). The inclusion of growth regulators in the culture medium has been successful in previous attempts to initiate cocoa embryos and/or embryogenic callus when using leaf (Litz, 1986), cotyledon (Aguilar *et al*., 1992), zygotic axes (Essan, 1977; Pence *et al*., 1980) and inflorescence explants (Li *et al*., 1998; Tan and Furtek, 2003) and thus were employed in this study. The various combinations of growth regulators gave rise to either white friable callus and/or translucent crystalline callus and/or brown soft callus (Figure 2.5a). These callus types when examined microscopically were revealed as non-embryogenic (Figure 2.5b); the cells were

irregular in shape and highly vacuolated. Explants placed on MS medium without the addition of growth regulators showed no response.

The percentage of explants producing callus was significantly different amongst treatments. Callus production was significantly higher, (at 65%; $p < 0.05$) when leaf explants were placed on medium incorporating 1.0 mg l^{-1} 2,4-D. Significantly lower percentages of explants produced callus when exposed to medium supplemented with 2,4-D in combination with either BAP or TDZ (Table 2.29).

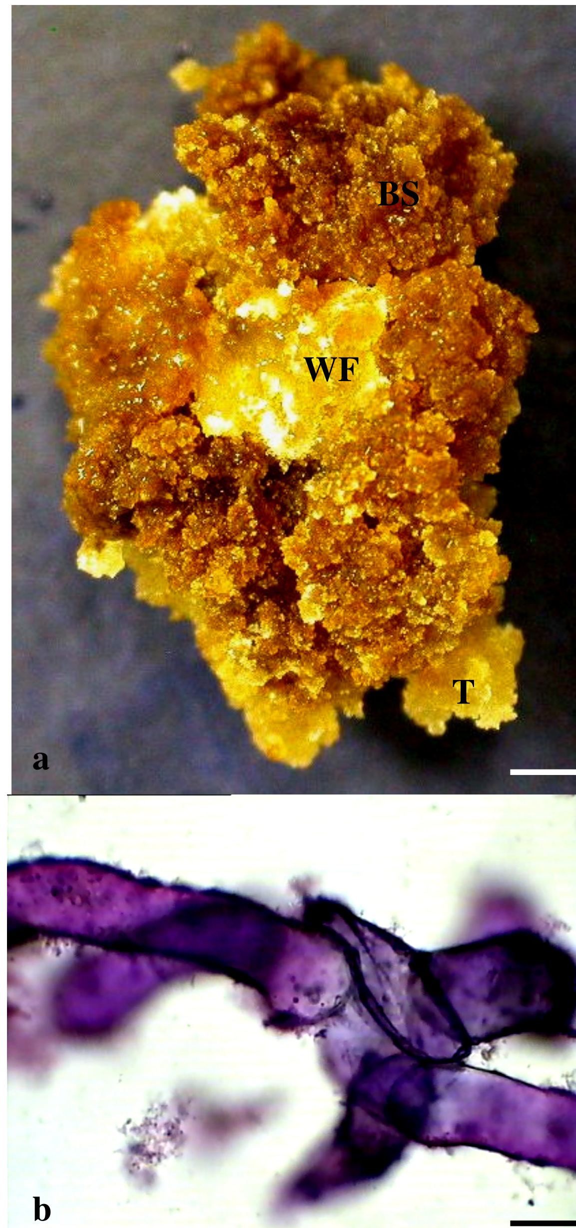


Figure 2.5: (a) The different types of callus formed by leaf explants placed on MS medium supplemented with 2,4-D alone, or with BAP or TDZ. BS-brown soft, WF-white flakes, T- translucent. Scale bar = 3 mm; (b) Different types of callus viewed microscopically revealed non-embryogenic cells. Scale bar = 0.1 mm

Table 2.29: Effect of 2,4-D or 2,4-D in combination with either BAP or TDZ on leaf segments of cocoa cultured on MS medium. **Types of callus: WF** = white friable callus, **T** = translucent crystalline callus and **BS** = brown soft callus. **Amount of callus scored as**: **(-)** = no callus, **(+) =** quarter of explant, **(++)** = half of explant, **(+++) =** three quarter of explant and **(++++)** = whole explant. n=50. Mean values represented by the same alphabetical letters are not significantly different (Chi-squared test, $p < 0.05$).

Growth regulator (mg l^{-1})			(%) explants producing callus	Type of callus	Amount Of Callus
2,4-D	TDZ	BAP			
-	-	-	0 [a]	-	-
0.5	-	-	25 [b]	WF T	+ +
1.0	-	-	65 [c]	WF T BS	++ + +
2.0	-	-	30 [b]	WF	+
3.0	-	-	25 [b]	WF BS	+ +
5.0	-	-	30 [b]	WF	+
1.5	-	0.1	15 [ab]	WF	+
1.5	-	0.5	20 [b]	T WF	+ +
1.5	-	1.0	5 [a]	WF	+
1.5	0.01	-	5 [a]	WF	++
1.5	0.05	-	5 [a]	T	+
1.5	0.1	-	0 [a]	-	-

Leaf explants cultured on DKW medium supplemented with 1.5 mg l^{-1} 2,4-D in combination with 0.1 or 1.0 mg l^{-1} TDZ, produced yellow/cream callus (Figure 2.6a) which was embryogenic (Figure 2.6b) (Table 2.30). Although DKW medium was initially developed for the *in vitro* propagation of walnut (Driver and Kuniyuki, 1984), it

has proved to be effective in inducing somatic embryogenesis from explants of a variety of species (Preece *et al*., 1995). The fact that DKW medium provides a significantly higher concentration of calcium, sulphur and magnesium compared with MS medium (Tan and Furtek, 2003), may be pivotal for cocoa somatic embryogenesis and further differentiation. All leaf explants placed on DKW medium incorporating 0.5 mg l^{-1} 2,4-D in combination with 0.01 mg l^{-1} TDZ produced callus which, however, was not embryonic. Use of DKW medium containing 2.0 g l^{-1} 2,4-D and 100 mg l^{-1} 2-iP, which was successful in generating globular embryos when using cocoa inflorescence explants (Tan and Furtek, 2003), was not successful with leaf explants in this study.

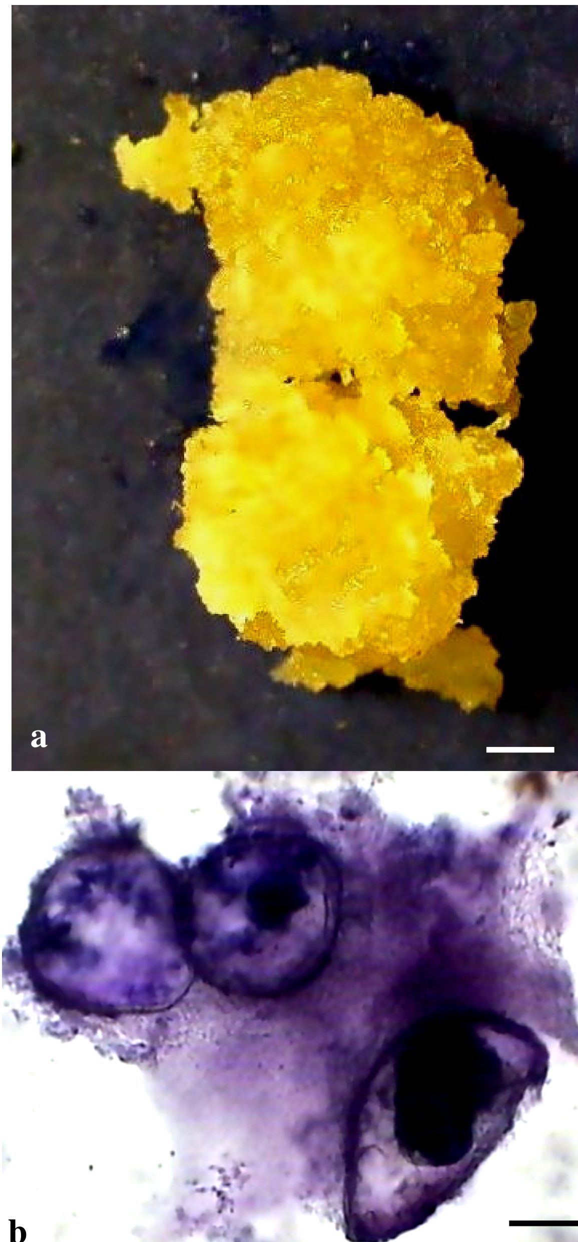


Figure 2.6: (a) Yellow/cream callus formed from both leaf and cotyledon explants, illustrated. Scale bar = 3 mm (b) Embryogenic cells from yellow/cream callus, Scale bar = 0.1 mm

Table 2.30: Effect of 2,4-D in combination with either 2-iP or TDZ in DKW medium on leaf segments of cocoa. **Types of callus: WF** = white friable callus, **BS** = brown soft callus, **T** = translucent callus and **Y =** yellow/cream compact callus. **Amount of callus scored as**: (-) = no callus, **(+) =** quarter of explant, **(++)** = half of explant, **(+++) =** three quarter of explant and **(++++)** = whole explant. n=50. Mean values represented by the same alphabetical letters are not significantly different (Chi-squared test, $p < 0.05$).

Growth regulator ($mg\ l^{-1}$)			% explants producing callus	Types of callus	Amount Of Callus
2,4-D	TDZ	2-iP			
-	-	-	5 [a]	WF	+
0.5	0.01	-	100 [c]	BS WF	++ +
0.5	0.05	-	40 [b]	WF T	+ +
0.5	1.0	-	50 [bc]	WF T	++ +
1.5	0.01	-	80 [bc]	T	++
1.5	0.1	-	50 [bc]	Y T	++ +
1.5	1.0	-	55 [bc]	Y T	+ +
1.5	-	1.0	75 [bc]	T WF	++ +
1.5	-	10	50 [bc]	T WF	+ +
1.5	-	50	0 [a]	-	-
2.0 $g\ l^{-1}$	-	100	0 [a]	-	-

As somatic embryos did not develop (even from the callus considered to be embryogenic) a protocol established by Li *et al.* (1998) for the induction of cocoa embryos from inflorescences, was investigated for leaf explants. Those authors induced

the development of cocoa somatic embryos from staminode explants by a three-step procedure, as follows: Explants were incubated on a primary callus induction medium, viz. DKW medium supplemented with 20 g l^{-1} glucose, 9.0 μ*M* 2,4-D and 22.7 n*M* TDZ, after which embryogenic calli were transferred onto a secondary callus growth medium, WPM supplemented with the same concentration of 2,4-D in combination with kinetin. Finally embryo development was facilitated on DKW medium devoid of plant growth regulators.

In this study, leaf explants producing embryogenic callus (on DKW medium supplemented with 1.5 mg l^{-1} 2,4-D in combination with either 0.1 or 1.0 mg l^{-1} TDZ [Table 2.30]) were sub-cultured on to secondary callus induction medium based on WPM with various concentrations of BAP and kinetin (Li *et al*., 1998). The results shown in Table 2.31 were recorded after explants had been maintained on the original induction medium for four weeks, followed by four weeks on secondary callus media, i.e. WPM with BAP or kinetin, with subsequent transfer to DKW medium devoid of growth regulators for a further four weeks. The formation of cream globular structures was observed on medium supplemented with 0.1 mg l^{-1} BAP; however, this occurred at a low frequency (Table 2.31). There was no significant difference in the percentage of explants producing callus irrespective of the primary callus induction medium tested.

Table 2.31: Responses of leaf explants on medium containing 1.5 mg l^{-1} 2,4-D in combination with 0.1 and 1.0 mg l^{-1} TDZ for 4 weeks followed by transfer on to one of seven different media based on WPM. n=20. Mean values represented by the same alphabetical letters within each column are not significantly different (Chi-squared test, $p < 0.05$).

Growth regulator (mg l^{-1})		1.5 mg l^{-1} 2,4 –D + 0.1 mg l^{-1} TDZ		1.5 mg l^{-1} 2,4 –D + 1.0 mg l^{-1} TDZ	
BAP	Kinetin	% explants producing yellow callus	% yellow callus producing globular	% explants producing callus	% yellow callus producing globular
-	-	70 ^{a}	0 ^{a}	60 ^{a}	0 ^{a}
0.1	-	45 ^{a}	20 ^{b}	35 ^{a}	0 ^{a}
0.5	-	40 ^{a}	0 ^{a}	40 ^{a}	0 ^{a}
1.0	-	50 ^{a}	0 ^{a}	50 ^{a}	0 ^{a}
-	0.1	40 ^{a}	0 ^{a}	40 ^{a}	0 ^{a}
-	0.5	40 ^{a}	0 ^{a}	50 ^{a}	0 ^{a}
-	1.0	50 ^{a}	0 ^{a}	45 ^{a}	0 ^{a}

The ratio of cytokinin to auxin used in tissue culture influences growth of roots, shoots or embryos depending on the explant as well as the species (George, 1993). Irrespective of the concentrations used, NAA in combination with BAP or TDZ had no effect on the formation of somatic embryos or adventitious shoots on cocoa leaf explants (Table 2.32).

Table 2.32: The effect of NAA in combination with BAP and TDZ on leaf explants using DKW medium. **Types of callus: WF** = white friable callus and **BS** = brown soft callus. **Amount of callus scored as: (-)** = no callus, **(+) =** quarter of explant, **(++)** = half of explant, **(+++) =** three quarter of explant and **(++++)** = whole explant. n=50. Mean values represented by the same alphabetical letters are not significantly different (Chi-squared test, $p < 0.05$).

Growth regulator ($mg\ l^{-1}$)			% explants producing callus	Types of callus	Amount Of Callus
NAA	BAP	TDZ			
1.0	3.0	-	50 [ab]	WF	++
0.5	1.0	-	66 [ab]	WF	++
0.1	0.5	-	40 [b]	BS	+
1.0	-	1.0	80 [a]	WF	++
0.5	-	0.5	76 [a]	BS WF	+ ++
1.0	-	0.1	80 [a]	BS WF	++ +

2.2.5.2 Somatic embryo induction via cotyledon explants

Cocoa cotyledon material to be used for the induction of somatic embryos was decontaminated via a three-step procedure (treatment 6) found to be the best in terms of significantly reducing contamination (Table 2.33). The inclusion of 70% ethanol into the decontamination protocol reduced bacterial contamination significantly, possibly by making permeable the waxy cuticle of cotyledonary explants, thus facilitating penetration of Hibitane and NaOCl into the tissue.

Table 2.33: Decontamination of cotyledon explants for somatic embryo induction. n=20. Mean values represented by the same alphabetical letters within each column are not significantly different (Chi-squared test, $p < 0.05$).

Treatment No.	Treatment	Time (min)	% Contamination	
			Fungal	Bacterial
1.	1% NaOCl	5	15 [a]	80 [c]
2.	1% NaOCl	10	10 [a]	70 [bc]
3.	1% Hibitane 1% NaOCl	5 10	10 [a]	65 [bc]
4.	1% Hibitane 1% NaOCl	10 10	5 [a]	50 [abc]
5.	70% EtOH 1% Hibitane 1% NaOCl	2 10 10	0 [a]	30 [ab]
6.	70% EtOH 2% Hibitane 1% NaOCl	2 10 10	0 [a]	15 [a]

Preliminary experiments using cotyledonary explants revealed that phenolics exudation constituted a major problem upon culture initiation; in an attempt to counteract this, explants were treated with two concentrations of citric acid, an anti-oxidant. Soaking both axillary bud and nodal explants in an anti-oxidant solution was found to be beneficial in counteracting phenolics exudation earlier in this study, as well as for *Prunus avium* microshoots (Vasar, 2003) and litchi fruit (Duan *et al*, 2007). A ten minute immersion in 2% citric acid significantly reduced the browning of the culture medium associated with phenolics exudation by cotyledonary explants ($p < 0.05$, Table 2.34). This pre-treatment was then routinely applied to cotyledonary explants, prior to any of the following manipulations.

Table 2.34: Effect of citric acid pre-treatment on cotyledon explants. n=20. Mean values represented by the same alphabetical letters are not significantly different (Chi-squared test, $p < 0.05$).

Citric acid concentration	Time	% explant browning
1%	5 min	100 [c]
	10 min	60 [b]
2%	5 min	30 [ab]
	10 min	5 [a]

Aguilar *et al*. (1992) obtained somatic embryos from cotyledon explants by culturing them on medium containing 3.0 mg l^{-1} BAP in combination with 1.0 mg l^{-1} NAA. In the present study, however, provision of NAA in combination with BAP or TDZ did not induce embryo production (Table 2.35). In addition, neither type of callus produced by the explants (white friable and brown soft) was embryogenic, nor was there any significant difference in the percentage of explants producing callus. The protocol used to generate somatic embryos from cotyledon explants by Aguilar *et al*. (1992) was not successful in this study. This may have been because the material used in the present study was of a different genotype compared with material used in that study: protocols established are usually species, and in most cases, genotype specific (Maximova *et al*., 2002).

Table 2.35: Effects of NAA in combination with BAP and TDZ in DKW medium on embryo formation. **Types of callus: WF** = white friable callus and **BS** = brown soft callus. **Amount of callus scored as: (-)** = no callus, **(+) =** quarter of explant, **(++)** = half of explant, **(+++) =** three quarter of explant and **(++++)** = whole explant. n=50. Mean values represented by the same alphabetical letters are not significantly different (Chi-squared test, $p < 0.05$).

Growth regulator (mg l^{-1})			% explants producing callus	Types of callus	Amount Of Callus
NAA	BAP	TDZ			
1.0	3.0	-	100 [a]	BS WF	+++ +
0.5	1.0	-	86 [a]	BS WF	+++ +
0.1	0.5	-	100 [a]	BS WF	++ +
1.0	-	1.0	100 [a]	BS	+++
0.5	-	0.5	80 [a]	BS WF	++ +
1.0	-	0.1	100 [a]	WF BS	++ +

Therefore, procedures used for leaf explants were applied to cotyledon explants including the three-step procedure (see above) established by Li *et al*. (1998). The effect of MS medium supplemented with 2,4-D and 2,4-D in combination with TDZ was investigated as these combinations gave rise to yellow/cream embryogenic callus when using leaf explants (see above). The inclusion of more than 2.0 mg l^{-1} 2,4-D into the culture media when using cotyledon explants was associated with low proportions of explants producing callus (Table 2.36). In no case, however, was embryogenic callus formed on the growth-regulator supplemented MS medium. Instead, non-embryogenic white friable, translucent crystalline, compact white and brown soft callus resulted.

Table 2.36: The effect of growth regulators in MS medium on cotyledon explants of cocoa. **Types of callus**: **WF** = white friable callus, **T** = translucent callus, **CW** = compact white callus, **BS** = brown soft callus. **Amount of callus scored as:** (-) = no callus, **(+) =** quarter of explant, **(++)** = half of explant and **(+++) =** three quarter of explant, **(++++)** = whole explant. n=50. Mean values represented by the same alphabetical letters are not significantly different (Chi-squared test, $p < 0.05$).

Growth regulator (mg l^{-1})		% explants producing callus	Types of callus	Amount of callus produced
2,4-D	TDZ			
0.5	-	66 ^{bcde}	WF	++
1.0	-	30 ^{abcd}	WF T	+ +
2.0	-	46 ^{abcde}	WF	+
3.0	-	24 ^{abc}	WF	+
5.0	-	10 ^{ab}	WF	+
10	-	6 ^{a}	T	+
1.0	0.005	74 ^{cde}	WF CW	++ ++
1.0	0.01	66 ^{bcde}	WF CW	++ +
1.0	0.05	66 ^{bcde}	WF CW BS	++ + +
1.0	0.1	90 ^{e}	T CW BS	+ + +
2.0	0.005	90 ^{e}	WF CW	++ ++
2.0	0.01	70 ^{cde}	WF CW	+ +
2.0	0.05	64 ^{bcde}	WF CW	+ +
2.0	0.1	80 ^{cde}	T CW WF	+ + +

Use of 2,4-D in combination with TDZ or 2-iP in DKW medium was beneficial in promoting the formation of yellow/cream nodular callus. This callus-type was embryogenic when 1.5 mg l^{-1} 2,4-D was combined with either 0.1 or 1.0 mg l^{-1} TDZ (Table 2.37). The protocol established by Tan and Furtek (2003) using DKW medium supplemented with 2.0 g l^{-1} 2,4-D and 100 mg l^{-1} 2-iP for inflorescence explants, did not induce embryogenesis from cotyledon explants in the present study (Table 2.37).

Table 2.37: Effect of selected growth regulators incorporated in DKW medium, on callus production by cocoa cotyledon segments. **Types of callus: WF** = white friable callus, **BS** = brown soft callus and **Y =** yellow/cream compact nodular callus. **Amount of callus scored as: (-)** = no callus, **(+) =** quarter of explant, **(++)** = half of explant and **(+++) =** three quarter of explant, **(++++)** = whole explant. n=40. Mean values represented by the same alphabetical letters are not significantly different (Chi-squared test, $p < 0.05$).

Growth regulator(mg l^{-1})			% explants producing callus	Types of callus	Amount Of Callus
2,4-D	TDZ	2-iP			
1.5	0.01	-	75 ^{bc}	BS WF	++ +
1.5	0.1	-	80 ^{bc}	Y WF	++ +
1.5	1.0	-	90 ^{c}	Y WF BS	++ + +
1.5	-	1.0	15 ^{a}	WF BS	++ +
1.5	-	10	40 ^{ab}	WF BS Y	++ + +
1.5	-	50	10 ^{a}	WF BS	+ +
2.0 g l^{-1}	-	100	50 ^{abc}	T	++

The exposure of cotyledon explants to the three step procedure as described by Li *et al.* (1998) gave rise to globular somatic embryos (Figure 2.7). The explants were initially incubated on 1.5 mg l^{-1} 2,4-D with 1.0 mg l^{-1} TDZ for four weeks with subsequent transfer to WPM with and without 1.0 mg l^{-1} kinetin, for a further four weeks with final incubation on DKW medium without growth regulators gave rise to globular embryos (Table 2.38). In addition, globular embryos formed on medium supplemented with 1.5 mg l^{-1} 2,4-D and 0.1 mg l^{-1} TDZ with subsequent transfer onto the secondary callus medium which contained either 1.0 mg l^{-1} BAP or 0.5 or 1.0 mg l^{-1} kinetin.

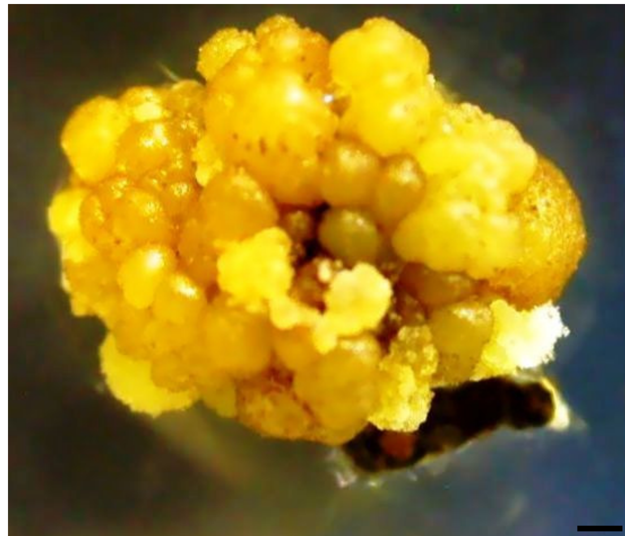


Figure 2.7: Globular embryos formed on cotyledon explant. Scale bar = 1 mm

Table 2.38: Effect of placing cotyledon explants on DKW medium containing 1.5 mg l^{-1} 2,4-D in combination with either 0.1 or 1.0 mg l^{-1} TDZ for 4 weeks and transferring explants onto seven different treatments based on WPM medium. n=50. Mean values represented by the same alphabetical letters within each column are not significantly different (Chi-squared test, $p < 0.05$).

Growth regulator (mg l^{-1})		1.5 mg l^{-1} 2,4-D + 0.1 mg l^{-1} TDZ		1.5 mg l^{-1} 2,4-D + 1.0 mg l^{-1} TDZ	
BAP	Kinetin	% explants producing yellow callus	% yellow callus producing globular embryos	% explants producing yellow callus	% yellow callus producing globular embryos
-	-	80^{b}	0^{a}	80^{a}	60^{a}
0.1	-	50^{a}	0^{a}	10^{c}	0^{b}
0.5	-	30^{a}	0^{a}	30^{bc}	0^{b}
1.0	-	50^{b}	30^{b}	50^{ab}	0^{b}
-	0.1	50^{b}	0^{a}	10^{c}	0^{b}
-	0.5	50^{b}	40^{b}	60^{a}	0^{b}
-	1.0	50^{b}	30^{b}	60^{a}	30^{a}

Due to time constraints, the globular embryos formed in this study could not be advanced towards germination. However, once this has been achieved, it is suggested that these structures could form suitable explants for cryopreservation.

2.3 Conclusion

The establishment of a long-term cryopreservation protocol for cocoa was not achieved in this study; however, steps towards developing a protocol were accomplished. Investigations of the various explants as potential specimens for cryopreservation revealed that axillary buds (section 2.2.1) that were smaller than 2 mm did not survive and larger buds regenerated very slowly *in vitro*. Minimal success was achieved in elongating buds if excised between 2 - 4 mm. These results suggested that axillary buds would not be amenable to cryopreservation because of the low regeneration capacity *in vitro*, as well as the relatively large size of the explant necessary for onwards development being too large for dehydration and cooling without lethal damage. However, a successful protocol for the proliferation, multiplication and rooting of shoots was achieved via greenhouse-derived nodal segments (Figure 2.8). This protocol can be used for the mass propagation of cocoa plantlets or shoots established could be utilised in minimal growth experiments, to facilitate a medium-term storage protocol for cocoa germplasm.

In vitro nodal segments as explants for cryopreservation (section 2.2.2) were selected as an alternate explant for investigation. Nodal segments have been used as explants for the long-term storage of *Antirrhinum microphyllum* (González-Benito *et al*., 1998) and *Dioscorea rotundata* (Quain *et al*., 2007). In this study, no survival of explants upon recovery from cooling after cryopreservation were obtained. Dehydration methods tested revealed that the tissue was highly desiccation-sensitive, and explants did not survive to sufficiently low water contents that would facilitate successful cooling. A regeneration system for *in vitro* nodal segments was however achieved, with successful plantlet formation from the explant (Figure 2.9). Cryopreservation of tropical woody species is considered challenging (Berjak and Pammenter, 2007). The potential explants are usually naturally at high water contents, and are highly desiccation-sensitive (Berjak and Pammenter, 2007; Quain *et al*., 2007). This was also found to be the case in the present study with *in vitro* nodal segments of cocoa. Therefore, the use of less complex, or less hydrated systems should be investigated. In this regard, apical meristems, clumps of meristem cells and perhaps even single cell cultures might prove to be suitable.

The present study also investigated the route of somatic embryogenesis, to generate embryos as explants for cryopreservation. Both leaf and cotyledon explants were used as primary explants, and were decontaminated successfully and cultured on various media. The best result was obtained when explants were subjected to a three-step culture process as established by Li *et al*. (1998) who used cocoa inflorescence explants. Globular stage embryos formed on explants maintained in DKW medium supplemented with 1.5 mg l^{-1} 2,4-D in combination with either 0.1 or 1.0 mg l^{-1} TDZ, with subsequent transfer onto a secondary callus medium containing WPM alone or in combination with either BAP or kinetin, and finally onto embryo development medium containing DKW medium devoid of plant growth regulators. In the present study, the globular embryos were not taken further but if plantlets can subsequently be established it is suggested that they may have the potential as explants for cryopreservation.

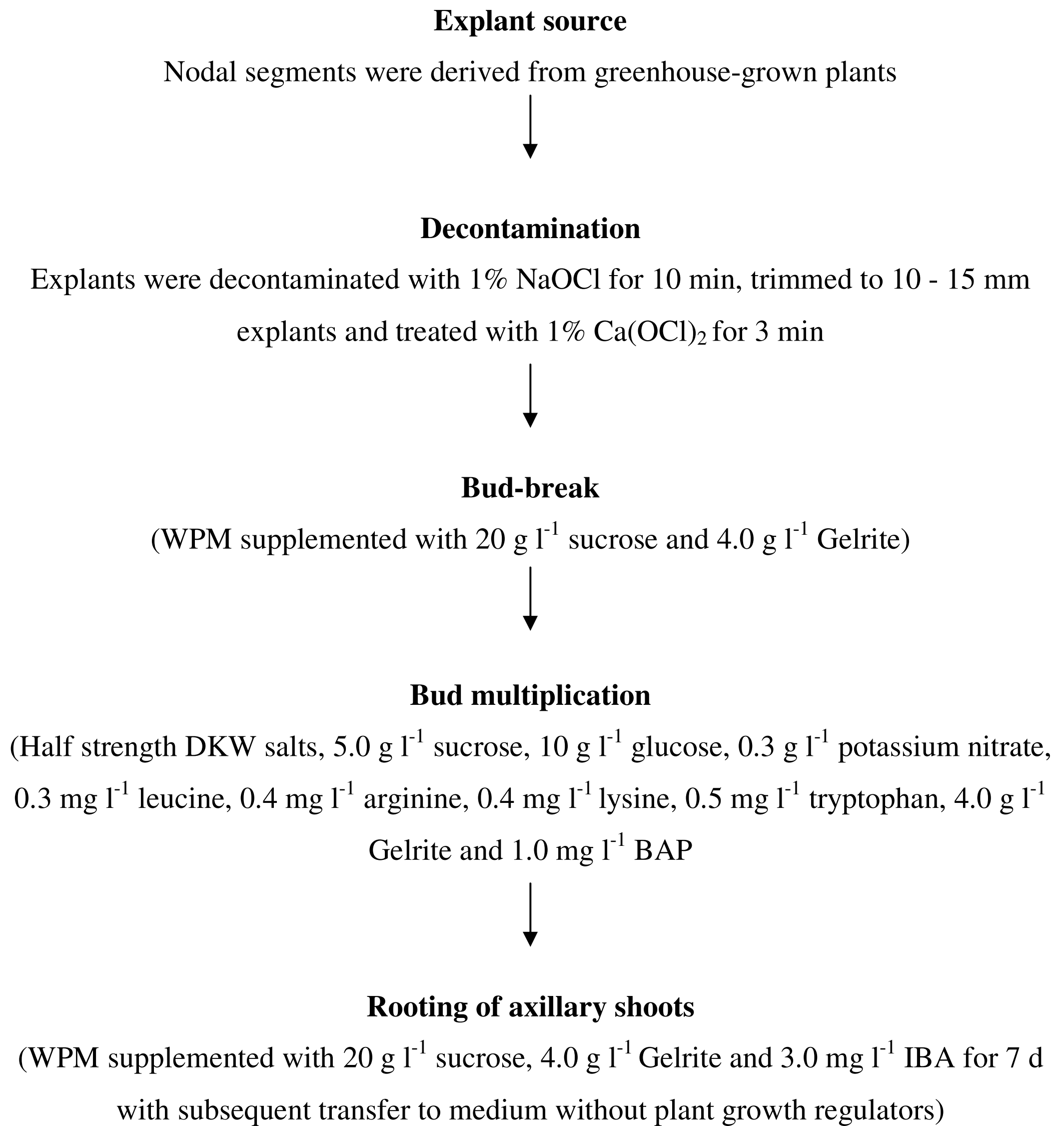
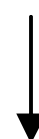


Figure 2.8: Propagation protocol developed for greenhouse-grown cocoa nodal segments

Explant source

Nodal segments were derived from *in vitro* established plants
(Zygotic axes germinated on MS medium supplemented with 30 g l^{-1}
sucrose and 8.0 g l^{-1} agar)

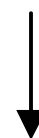


Pre-treatment and Decontamination

(Explants were pre-treated with 0.5% ascorbic acid for 10 min followed by
5 min in 1% $Ca(OCl)_2$ with rinses in sterile water after each step)

Bud-break

(Half-strength DKW salts, 5.0 g l^{-1} sucrose, 10 g l^{-1} glucose, 0.3 g l^{-1} potassium nitrate,
0.3 mg l^{-1} leucine, 0.4 mg l^{-1} arginine, 0.4 mg l^{-1} lysine, 0.5 mg l^{-1} tryptophan and 4.0 g
l^{-1} Gelrite)



Bud multiplication

(Half-strength DKW salts, 5.0 g l^{-1} sucrose, 10 g l^{-1} glucose, 0.3 g l^{-1} potassium nitrate,
0.3 mg l^{-1} leucine, 0.4 mg l^{-1} arginine, 0.4 mg l^{-1} lysine, 0.5 mg l^{-1} tryptophan, 4.0 g l^{-1}
Gelrite and 0.5 mg l^{-1} BAP

Rooting of axillary shoots

(WPM supplemented with 20 g l^{-1} sucrose, 4.0 g l^{-1} Gelrite and 3.0 mg l^{-1} IBA for 7 d
with subsequent transfer to medium without plant growth regulators)

Figure 2.9: Propagation protocol developed for *in vitro*-derived cocoa nodal segments.

CHAPTER 3: STUDIES ON *Barringtonia racemosa*

Barringtonia racemosa, as discussed earlier, grows best in swampy areas, and like cocoa, produces recalcitrant seeds. The interior of the seed is comprised virtually entirely of a large hypertrophied axis which cannot be used as the explant for cryopreservation (see Chapter 1). Therefore, investigations reported in this chapter entailed developing protocols for the development of shoots or somatic embryos that could provide potential explants for cryopreservation, so facilitating the long-term conservation of the genetic resources of this species. *Barringtonia racemosa* is not only medicinally important (Khan *et al*., 2001; Deraniyagala *et al*., 2002) but also belongs to the Lecythidaceae, the same family as *Bertholletica excelsa* (Brasil nut): thus, any success in developing micropropagation or conservation protocols could potentially be applied to the latter, which is a valuable commercial crop on which such studies appear to be minimal (Silvertown, 2004).

3.1 Materials and Methods

Fruits of *B. racemosa* were collected from the University of KwaZulu-Natal (Howard College), Durban and the La Mercy area just to the north of Durban. Upon arrival in the laboratory, the fruits were soaked in 1% sodium hypochlorite (NaOCl) for 10 min followed by three rinses with tap water, blotted dry and then stored on plastic trays at 16˚C.

3.1.1 Seedling establishment

Hypertrophied axes were excised and surface-decontaminated using a three-step procedure. Firstly, axes were soaked in an aqueous fungicidal cocktail consisting of 0.33 ml 100 ml^{-1} Celest (Syngenta, South Africa) and 0.08 g 100 ml^{-1} Heritage (Syngenta, South Africa) for 2 h. Axes were subsequently soaked in 2% Hibitane®, which is bacteriocidal, for 10 min, followed by 1% NaOCl for 10 min, with three rinses in sterile distilled water after each step. Contamination percentages were recorded after three weeks on MS medium. Different basal media as well as different salt concentrations were tested as germination media. Two axes were placed in each of ten 500-ml Consol® bottles with 100 ml of medium per bottle. After eight weeks in culture,

percentage contamination, percentage of explants producing both roots and shoots and number of shoots per explant were recorded.

3.1.2 Shoot multiplication via embryonic axes

Explants were decontaminated as described above, but to reduce contamination all explants were trimmed such that only the central portion or core of the axis was cultured. Axis cores were placed on MS multiplication medium containing 30 g l^{-1} sucrose, 8.0 g l^{-1} bacteriological agar, and BAP or 2-iP (0.1, 1.0, 10 and 50 mg l^{-1}). Explants were placed on 30 ml of multiplication medium in 90-mm Petri dishes and incubated in the dark at 25±2˚C. After eight weeks in culture percentage contamination, percentage of explants producing shoots and number of shoots per explant were recorded. Shoots were elongated and rooted on MS medium devoid of growth regulators.

3.1.3 Somatic embryogenesis

For the induction of somatic embryos two types of explants were investigated, viz immature inflorescences and zygotic axis segments. Immature petals from *B. racemosa* inflorescences were decontaminated by soaking for 5 h in 50 µl 100 ml^{-1} Orius 20 EW (Irvita Plant Protection N.V, Netherlands) thereafter with 2% Hibitane for 10 min followed by 1% NaOCl for 10 min. After each treatment, explants were rinsed in sterile distilled water three times. Although the decontamination protocol was effective in eliminating 90% of the contamination, it resulted in tissue death (see Results and Discussion). Therefore, the use of inflorescence explants was precluded from further studies.

Zygotic axes were decontaminated as described in 3.1. Axes were subsequently sliced into 2-mm-thick longitudinal sections under sterile conditions and placed on MS medium supplemented with different growth regulators (Table 3.1). The effect of NAA in combination with BAP (CIa-1 - CIa-5[*]), various concentrations of 2,4-D (CIa-6 - CIa-10) and 2,4-D in combination with BAP (CIa-11 - CIa-13) was investigated.

[*] Medium codes (ref. Results and Discussion) given in parentheses – see Table 3.1

Thereafter the optimum concentration of 2,4-D which gave rise to embryogenic callus was tested in combination with BAP and thidiazuron (TDZ) (CIb-1 - CIb-6).

Table 3.1: The different growth regulators tested for callus induction. Media for all treatments contained MS medium supplemented with 30 g l^{-1} sucrose and 8.0 g l^{-1} agar.

Medium	Growth regulator (mg l^{-1})			
code	2,4-D	NAA	BAP	TDZ
CIa	-	-	-	-
CIa-1	-	1.0	1.0	-
CIa-2	-	0.5	1.0	-
CIa-3	-	0.1	1.0	-
CIa-4	-	1.0	2.0	-
CIa-5	-	0.1	2.0	-
CIa-6	0.5	-	-	-
CIa-7	0.7	-	-	-
CIa-8	1.0	-	-	-
CIa-9	2.0	-	-	-
CIa-10	3.0	-	-	-
CIa-11	0.5	-	0.1	-
CIa-12	0.7	-	1.0	-
CIa-13	1.0	-	0.2	-
CIb-1	2.0	-	0.1	-
CIb-2	2.0	-	0.5	-
CIb-3	2.0	-	1.0	-
CIb-4	2.0	-	-	0.01
CIb-5	2.0	-	-	0.05
CIb-6	2.0	-	-	0.1

In addition, the effect of pre-treating explants with liquid medium prior to incubation on solid medium was investigated for the formation of somatic embryos. Twenty embryo segments were placed in a RITA® vessel with 200 ml MS-containing liquid medium supplemented with 30 g l^{-1} sucrose, 2.0 mg l^{-1} 2,4-D and 0.1 mg l^{-1} BAP. The embryo

explants were flushed every 30 min for 30 sec with the liquid medium and were incubated for periods of 24 h, 3 d and 7 d. After incubation in the RITA vessels, explants were plated on MS medium solidified with 8.0 g l^{-1} agar devoid of plant growth regulators or plated on MS medium supplemented with 30 g l^{-1} sucrose, 2.0 mg l^{-1} 2,4-D, 0.1 mg l^{-1} BAP and 8.0 g l^{-1} agar.

All media were adjusted to pH 5.6 - 5.8 prior to autoclaving at 100 Kpa at 121˚C for 20 min. For each treatment ten 90-mm Petri dishes with five explants per Petri dish placed on 30 ml of induction medium were used, and incubated in the dark at 25±2˚C. After six weeks, all cultures were screened and explant responses to each callus induction medium were assessed in terms of the number of explants producing callus, the type of callus formed as well as the amount of each callus type produced, which was scored using (+) symbols, each symbol being representative of quarter of the explant. Therefore, four symbols meant the upper surface of the whole explant had produced callus.

3.1.4 Processing for microscopy: Paraffin wax embedding of globular structures

Globular structures formed on MS medium supplemented with 30 g l^{-1} sucrose, 1.0 g l^{-1} casein hydrolysate, 2.0 mg l^{-1} 2,4-D, 0.1 mg l^{-1} BAP and 8.0 g l^{-1} agar, were wax embedded. Samples had been fixed in FAA (10 ml formalin, 5 ml glacial acetic acid, 50 ml 95% ethanol, 35 ml distilled water) for 24 h at room temperature, after which they were dehydrated in a butanol:ethanol:distilled water series (Table 3.2).

Table 3.2: Dehydration of globular structures using a butanol:ethanol:distilled water series

Butanol (ml)	Ethanol (95%) (ml)	Distilled water (ml)	Time (min)
10	20	70	30
15	25	60	30
25	30	45	30
40	30	30	30
55	25	20	45
70	20	10	60
85	15	0	90
100	0	0	Overnight

The samples were then infiltrated with paraffin wax using a series of wax/butanol mixtures (Table 3.3).

Table 3.3: Infiltration of globular structures using wax/butanol series

Wax (ml)	Butanol (ml)	Time (hours)
25	75	2
50	50	3
75	25	3
100 (molten)	0	Overnight at 55˚C

After the overnight infiltration the samples were immersed in 100% fresh molten wax prior to proceeding with block mould preparation. The wax was melted in an oven at 70˚C, poured into plastic moulds (Peel-A-Way®, Polysciences), the sample positioned at the base of the mould using a pair of forceps and the wax solidified at room temperature. The wax blocks were sectioned using an American optical 820 rotary microtome, and sections placed on drops of water on slides pre-treated with Haupt's adhesive (1.0 g gelatin, 100 ml water, 15 ml glycerol and 2.0 g phenol crystals). The gelatin and water were mixed in a beaker and placed in a water bath at 30˚C until the gelatin had melted; thereafter the glycerol and phenol crystals were added. Slides were

coated with a thin layer of adhesive using a paint brush, excess adhesive allowed to run off, and then left to dry before use.

The sections on the slides were dried down, dewaxed in three 10 min changes of xylene, and then rehydrated through a series of ethanol solutions (100%, 75%, 50% and 30%) for 5 min at each concentration. Slides were then rinsed in distilled water and stained with 0.1% Toluidine Blue (pH 7) made up with phosphate buffer saline (PBS).

To enhance the formation, and promote further development, of globular structures formed from zygotic axis segments, the use of different size axes (as a measure of maturity) was investigated. Three size classes of zygotic axes (Table 3.4) were decontaminated as described in 3.1 and placed on the induction medium which comprised MS medium supplemented with 30 g l^{-1} sucrose, 2.0 mg l^{-1} 2,4-D, 0.1 mg l^{-1} BAP and 8.0 g l^{-1} agar. After the size of the axis was optimised for callus induction, the inclusion of different concentrations of sucrose (30, 60, 90 and 120 g l^{-1}) and casein hydrolysate (0.1, 0.5, 1.0 g l^{-1}) to the induction medium was investigated. Five explants were plated on 30 ml of medium in 90-mm Petri dishes. After six weeks on the induction medium, the percentage of explants producing embryos was recorded and the number of globular and heart shaped embryos forming was scored using (+) symbols. Each symbol was representative of the following numbers of embryos forming: **+** (1-5 embryos), **++** (6-10 embryos), **+++** (11-20 embryos), **++++** (> 20 embryos).

Table 3.4: The different size zygotic axis explants tested.

Embryo size	Mass (g)	Length (mm)	Breadth (mm)
Class 1	0.33	12.63	7.20
Class 2	2.97	17.20	10.88
Class 3	20.44	36.30	31.33

3.1.5 Somatic embryo maturation

Explants were incubated on the induction medium (MS medium, 30 g l^{-1} sucrose, 1.0 g l^{-1} casein hydrolysate, 2.0 mg l^{-1} 2,4-D, 0.1 mg l^{-1} BAP and 8.0 g l^{-1} agar) for different periods (1 - 4 weeks), then placed on half- or full-strength MS medium devoid

of plant growth regulators, and containing 30 g l^{-1} sucrose, 1.0 g l^{-1} casein hydrolysate and 8.0 g l^{-1} bacteriological agar (Media S1 and S2, respectively). Thereafter medium S2 was supplemented with 0.1 or 1.0 mg l^{-1} BAP (Media S3 and S4), 0.1 or 1.0 mg l^{-1} ABA (Media S5 and S6). After four weeks, all cultures were screened and the explant response to each of these secondary callus media was assessed. The number of explants producing callus, the type of callus formed as well as the amount of callus produced, which was scored using (+) symbols, being representative of quarter of the explant.

Other conditions tested included placing explants that had been incubated for six weeks on the induction medium, on secondary callus media consisting of MS medium supplemented with three different auxins (NAA, IAA and 2,4-D) each at 0.1 or 0.7 mg l^{-1} in combination with either 0.1 or 1.0 mg l^{-1} BAP or kinetin. Explants with globular structures were also desiccated for 3, 6, 12 and 24 h between two pieces of sterile filter paper in 90-mm sterile Petri dishes, after which they were plated on MS medium without growth regulators. Another parameter tested was temperature, where explants were subjected to cold treatment. Explants producing globular stage embryos were incubated at 4˚C for 4, 8, 24, 48, 96 and 168 h and then placed in the dark at 25±2˚C. After six weeks on the secondary medium the percentage of explants producing embryos was recorded and the number of globular and heart shaped embryos forming was scored as follows: **+** (1-5 embryos), **++** (6-10 embryos), **+++** (11-20 embryos), **++++** (> 20 embryos).

Media were adjusted to pH 5.6 - 5.8 with either 0.1 M HCl or 0.1 M NaOH for all experiments and autoclaved at 121˚C at 100 Kpa for 20 min.

3.1.6 Photography and Microscopy

All photographs were taken with a Nikon Coolpix® 4500 digital camera. Embryogenic and non-embryogenic cells as well as sections were stained with 0.1% Toluidine Blue in PBS buffer, pH 7.4 and viewed using a Nikon Biphot® Photomicroscope.

3.1.7 Data analysis

Statistical analyses were performed using SPSS 15.0 for windows. The Scheffé's multiple range test (one-way ANOVA) was used for parametric data. The chi-square test was used for non-parametric binomial data. The level of significance for all statistical analyses performed was set at $p < 0.05$. The mean value for each treatment was assigned an alphabetical symbol. Mean values that do not share the same letter are recognised as being significantly different.

3.2 Results and Discussion

3.2.1 The development of *in vitro* shoots

The establishment of shoots as potential explants for cryopreservation was via germination of embryonic axes *in vitro*. *Barringtonia racemosa* zygotic axes are hypertrophied and very large, complicating *in vitro* culture compared with other recalcitrant seeds where the axis makes up only a small fraction of the entire mass or volume (Berjak *et al*., 1989). The first part of the present study dealt with establishing a decontamination protocol for embryonic axes prior to culture initiation.

The use of fungicides alone or in combination was tested for various treatment times. The best treatment, which resulted in significantly the lowest ($p < 0.05$) fungal contamination, was an aqueous fungicidal cocktail consisting of Celest and Heritage applied for a period of 2 h (Treatments 14 and 15, Table 3.5). Celest or Heritage applied alone did not have a significant effect in reducing fungal contamination compared with the combined effect of these fungicides. The active ingredients in Celest and Heritage are fludioxonil and azoxystrobin, respectively. Fludioxonil belongs to the phenylpyrrole family, and acts by inhibiting MAP kinase proteins the disruption of which results in dehydration and rupture of the fungal cells ascribed to erratic or non-existent osmotic potential regulation (Montgomery and Paulsrud, 2006). Azoxystrobin belongs to the strobilurin family, which as a group, have wide applicability against an array of pathogens (Bartlett *et al*., 2001). According to those authors, strobilurins act by inhibiting mitochondrial respiration in fungi, which disrupts the energy cycle by halting the production of ATP. The combination of Celest and Heritage has been found to be efficient in eliminating various pathogens associated with recalcitrant seeds of other species at the Plant Germplasm Conservation Research Laboratory at the University of KwaZulu-Natal (Reddy *et al*., 2007), with which the findings of this study concur.

After a reduction in fungal contamination, there was an increase in bacterial proliferation. It is suggested that the fungi exerted an antibiotic effect on the bacteria and once the fungi were eliminated, bacteria could proliferate. The inclusion of Hibitane in the decontamination protocol for 10 min after treatment with Celest and Heritage (Treatment 15) reduced bacterial contamination considerably (Table 3.5). Chlorhexidine

gluconate, the active ingredient in Hibitane, has wide efficacy against an array of bacteria, fungi and viruses and is widely used in the medical field (Anon, 2007).

Table 3.5: Different treatments used for decontamination of *Barringtonia racemosa* embryonic axes. Treatments 3 - 14 were followed by 10 min soak in 1% NaOCl and Treatment 15 utilised 2% Hibitane for 10 min followed by 10 min in 1% NaOCl with three rinses in sterile distilled water after each step. n=20. Mean values represented by the same alphabetical letters within each column are not significantly different (Chi-squared test, $p < 0.05$).

No.	Treatment	Treatment Time (min)	Contamination (%)	
			Fungal	Bacterial
1	1% NaOCl	5	100^{a}	-
2	1% NaOCl	10	100^{a}	-
3	Previcur (2.5 ml l^{-1}) + Impact (0.5 ml l^{-1})	30	60^{bc}	40^{ab}
4		60	70^{b}	30^{a}
5		120	50^{bc}	30^{a}
6	Celest (0.33 ml 100 ml^{-1})	30	60^{bc}	40^{ab}
7		60	50^{bc}	50^{abc}
8		120	50^{bc}	40^{ab}
9	Heritage (0.08 g 100 ml^{-1})	30	70^{b}	30^{a}
10		60	50^{bc}	50^{abc}
11		120	40^{bc}	50^{abc}
12	Celest (0.33 ml 100 ml^{-1}) and Heritage (0.08 g 100 ml^{-1})	30	30^{c}	70^{bc}
13		60	30^{c}	70^{bc}
14		120	5^{d}	80^{c}
15	Celest, Heritage followed by Hibitane	120	5^{d}	5^{d}

After decontamination (using Treatment 15), germination (root and shoot production) of *B. racemosa* whole axes occurred after 28 days on quarter-strength MS medium and

after 35 days on half- and full-strength MS medium with no germination occurring on WPM (Table 3.6). Although the chi-squared test showed no significant difference ($p > 0.05$) in percentage germination amongst the different strengths of MS medium tested, there was a significant difference in the average number of shoots produced per explant for these treatments (Table 3.6, $p < 0.05$). Embryonic axes placed on full-strength MS medium produced 1.3 shoots per explant while those germinated on quarter-strength MS produced 2.3 shoots per explants (Figure 3.1), the latter therefore being selected as the best medium for germination.

Even though explants were decontaminated, and only 5% appeared bacterially contaminated immediately following treatment, using the protocol Treatment 15 (above), 40 - 53% whole axes cultured for germination showed bacterial contaminants (Table 3.6). The high contamination frequency is suggested to have contributed to the low percentage of germination recorded for zygotic axis explants on the MS-based media (Table 3.6). It is probable that the Hibitane did not penetrate sufficiently to affect bacterial inoculum located deep within the embryo tissues.

Table 3.6: The effects of different basal media on germination of *B. racemosa* embryonic axes. **MS -** Murashige and Skoog, 1962, **WPM** - Woody Plant Medium, Lloyd and McCown, 1981. n=15. Mean values represented by the same alphabetical letters are not significantly different within columns (Chi-squared test, $p < 0.05$).

Basal medium	% Germination	Avg. no. of shoots produced/ explant	Time taken for germination (days)	% Contamination
MS	56 [a]	1.3 [b]	35	43 [a]
½ MS	30 [a]	1 [b]	35	53 [a]
¼ MS	40 [a]	2.3 [a]	28	50 [a]
WPM	0 [b]	0 [c]	0	50 [a]

Figure 3.1: **Multiple shoot formation from *B*. *racemosa* zygotic axis germinated on quarter-strength MS medium. Scale bar = 3 mm**

Germination of axes using only the central core region and eliminating the contaminated exterior reduced the percentage of contaminated explants, and 60% of the explants germinated on quarter-strength MS medium (results not shown). Therefore, when culturing axes onto medium containing BAP or 2-iP to promote adventitious shoot formation or shoot multiplication, the explants were trimmed such that only the axis core was cultured.

The inclusion of BAP or 2-iP in the culture medium had a significant effect on shoot multiplication (Table 3.7). Media supplemented with 10 mg l^{-1} BAP or 2-iP produced an average of 1.7 and 2 shoots per axes, respectively. Exposure to 50 mg l^{-1} BAP or 2-iP, however, resulted in no shoot production, which has also been shown for *Zantedeschia aethiopica* axes (Ngamau, 2001). There was no significant difference in the percentage of explants producing shoots across the treatments as well as among media supplemented with BAP or 2-iP (Table 3.7, $p > 0.05$). The time required for shoot formation was however, affected by the inclusion of PGRs in the medium. Explants

exposed to BAP-containing medium took a considerably shorter period for shoot formation compared with those on 2-iP containing medium (Table 3.7).

Callus formation occurred only on the surface of explants placed on media supplemented with 0.1 and 10 mg l^{-1} 2-iP, no callus forming on explants exposed to different concentrations of BAP (Table 3.7). This was somewhat unexpected as the inclusion of BAP has been found to be beneficial for indirect formation of somatic embryos when zygotic axes of a variety of species were utilised as the primary explant (e.g. George, 1996); however, this PGR had no effect in the present study on either shoot multiplication or callus formation.

Table 3.7: The effects of two cytokinins, BAP and 2-iP, on shoot formation from embryonic axis cores. Explants were placed on MS medium supplemented with 30 g l^{-1} sucrose and various concentrations of BAP or 2-iP. **(+)** = callus, **(-)** = no callus. n=20. Mean values represented by the same alphabetical letters within each column are not significantly different (Chi-squared test, $p < 0.05$).

Growth Regulator (mg l^{-1})		% explants producing shoots	Avg. no. of shoots produced/ explant	Callus formation	Time taken to form shoots (days)
2-iP	BAP				
0.1		35^{a}	1.3^{ab}	+	56
1.0		40^{a}	1^{b}	-	42
10		45^{a}	1.7^{a}	+	42
50		0^{b}	0^{c}	-	0
	0.1	30^{a}	1^{b}	-	28
	1.0	25^{a}	1^{b}	-	28
	10	35^{a}	2^{a}	-	21
	50	0^{b}	0^{c}	-	0

The use of *in vitro* generated shoots as explants for cryopreservation was decided against because of their thick and leathery nature. In addition, the lack of success

obtained with cocoa vegetative tissue as well as that of other tropical woody species (see Chapter 2), did not encourage the use of such material for cryogen exposure in the present study. Therefore, it was decided that somatic embryos might be more feasible specimens for conservation of *B. racemosa* germplasm.

3.2.2. The development of a somatic embryogenesis protocol

Although less than ideal in terms of genetic diversity conservation, somatic embryos offer many benefits in terms of clonal micropropagation, genetic engineering, cryopreservation and synthetic seed formation (Merkle *et al*., 1990; George, 1996). For the induction of *B. racemosa* somatic embryos, two types of explants were investigated, i.e. immature inflorescences and the hypertrophied zygotic axis. As discussed earlier (see Chapter 1), the choice of the primary explant for the induction of somatic embryos is a critical factor since some explants have a greater capacity for embryogenesis than do others (Merkle *et al*., 1990).

Somatic embryo development proceeds from the early globular to the globular, heart, torpedo, and cotyledonary stages, finally resulting in embryo germination (George, 1996). Although the initial stages are achieved readily, germination of somatic embryos and establishment of viable plantlets is suggested to be challenging (Junaid *et al*., 2006). This was the case with *B. racemosa*, as discussed below.

3.2.3 Somatic embryo induction via immature inflorescences

Immature inflorescence explants have been used to initiate somatic embryos because of their morphogenetic capabilities. *Barringtonia racemosa* inflorescences were readily available seasonally, which allowed for experimentation. However, one of the major problems experienced with culture initiation was contamination. Inflorescence explants had a higher fungal load than embryonic axes as discovered in preliminary experiments, therefore they were exposed to longer treatment periods. The combination of Celest and Heritage as used for decontaminating *B. racemosa* axes (see 3.2.1) was not effective in eliminating contaminants associated with inflorescence explants since 100% contamination persisted after a 24 h treatment period (Table 3.8). Therefore, a harsher fungicide was used, viz. Orius. The active ingredient in Orius is tebuconazole, which is

recognised as a demethylation-inhibitor fungicide (DMI) known to be effective in diminishing the incidence of many groups of pathogens (Holb and Schnabel, 2007). Studies have shown that tebuconazole has one of the best curative activities among triazoles (Schofl and Zinkernagel, 1997; Homdork *et al.*, 2000; Ferreira *et al.*, 2008; Holb and Schnabel, 2007).

Two concentrations of Orius were tested, 50 and 250 µl 100 ml^{-1}, for three different incubation durations. The percentage contamination was significantly reduced after treatment for an hour with 250 µl 100 ml^{-1} Orius compared with the 1 h treatment using 50 µl 100 ml^{-1} Orius (Table 3.8). There was no significant difference between the 3 or 5 h treatments for either concentration of Orius tested.

Inflorescence explants were plated on MS medium with a range of growth regulators. However, very few explants produced callus (results not shown). This was attributed to the decontamination protocol that resulted in tissue death, while a measure of contamination persisted. Attempts to reduce contamination by halving inflorescences or slicing them into quarters prior to decontamination, resulted in lower contamination frequencies; however, explants showed the same response, viz. no survival (results not shown). Therefore, inflorescence explants were not used in subsequent experiments.

Table 3.8: Decontamination procedures used for *B. racemosa* inflorescence explants. Treatments 1 - 4 were followed by 1% Hibitane for 10 min and then 1% NaOCl for a further 10 min while treatments 5 - 10 were treated with 2% Hibitane for 10 min followed by 1% NaOCl for 10 min with three rinses in sterile distilled water after each treatment. Explants were plated on MS medium supplemented with 30 g l^{-1} sucrose and 8.0 g l^{-1} agar. n=20-40. Mean values represented by the same alphabetical letters within columns are not significantly different (Chi-squared test, $p < 0.05$).

No.	Treatment	Treatment Time	Contamination (%)	
			Fungal	Bacterial
1	Celest (0.33 ml 100 ml^{-1}) + Heritage (0.08g 100 ml^{-1})	1 h	100 [a]	-
2		3 h	100 [a]	-
3		6 h	100 [a]	-
4		24 h	100 [a]	-
5	Orius (50 µl 100 ml^{-1})	1 h	100 [a]	-
6		3 h	55 [b]	40 [a]
7		5 h	15 [c]	30 [a]
8	Orius (250 µl 100 ml^{-1})	1 h	60 [b]	20 [a]
9		3 h	40 [bc]	25 [a]
10		5 h	10 [c]	15 [a]

3.2.4 Somatic embryo induction via embryonic axes

The induction of somatic embryos relies on the combination of various factors which act together to enhance the formation of embryogenic cells from somatic cells (Namasivayam, 2007). The basal salt concentration, together with the incorporation of growth regulators play an interactive role in forming somatic embryos (Carman, 1990; Razdan, 2003). In the present study MS medium (Murashige and Skoog, 1962) was selected as there have been numerous reports where it was used effectively in producing somatic embryos when zygotic axes were the primary explant material (e.g. George, 1996; Wu *et al.*, 2007). There was, however, no response when zygotic axis segments

were placed on MS medium devoid of PGRs (Table 3.9). The addition of auxin to the culture medium is generally essential for embryo initiation whether committed or uncommitted tissues are used as the primary explant (Merkle *et al*., 1990; Razdan 2003). Mostly, 2,4-D is incorporated in the embryo induction medium but other auxins, for example NAA, have also been used (e.g. George, 1996; Razdan 2003).

In the present study, both 2,4-D and NAA were tested. The exposure of embryo explants to the different growth regulators as well as different concentrations resulted in various responses, the most divergent being the percentage of explants producing callus (Table 3.9). Only low percentages of *B. racemosa* 2-mm-thick axis segments exposed to NAA in combination with BAP produced callus, which was white and flaky in appearance (Figure 3.2a) and not embryogenic (Figure 3.2b). It is well known that embryogenic cells forming somatic embryos from various plant systems such as sugarcane (Ho and Vasil, 1983), alfalfa (Dos Santos *et al*., 1983), pearl millet (Taylor and Vasil, 1996), and cork oak (Puigderrajols *et al*., 2001) as reviewed by Namasivayam (2007) are characteristically small and isodiametric with a large, densely staining nuclei and high nucleus:cytoplasm volume ratio. However, the cells forming white flaky callus were relatively large, irregular and highly vacuolated (Figure 3.2b).

Table 3.9: The effect of MS medium containing different growth regulators for somatic embryo induction on 2-mm-thick longitudinal segments of embryonic axes. **Types of callus: WF** - White flakes, **T -** Translucent, **Y** - Yellow, **GS** - Globular structures. **Amount of callus scored as**: (-) = no callus, **(+) =** quarter of explant, **(++)** = half of explant, **(+++) =** three quarter of explant and **(++++)** = whole explant. n=50. Mean values represented by the same alphabetical letters are not significantly different (Chi-squared test, $p < 0.05$).

Medium Code	% explants producing callus	Types of callus	Amount Of Callus
CIa	0 [d]	-	-
CIa-1	0 [d]	-	-
CIa-2	16 [c]	WF	++
CIa-3	16 [c]	WF	++
CIa-4	0 [d]	-	-
CIa-5	16 [c]	WF	++
CIa-6	100 [a]	WF T	++ +
CIa-7	100 [a]	WF T	++ +
CIa-8	86 [ab]	T Y GS	++ + +
CIa-9	100 [a]	WF Y GS	+ ++ +
CIa-10	70 [b]	T WF	++ +
CIa-11	80 [ab]	T	+++
CIa-12	100 [a]	WF T	++ +
CIa-13	100 [a]	T WF	++ +

Contamination levels were very low and therefore not shown in the table of results.

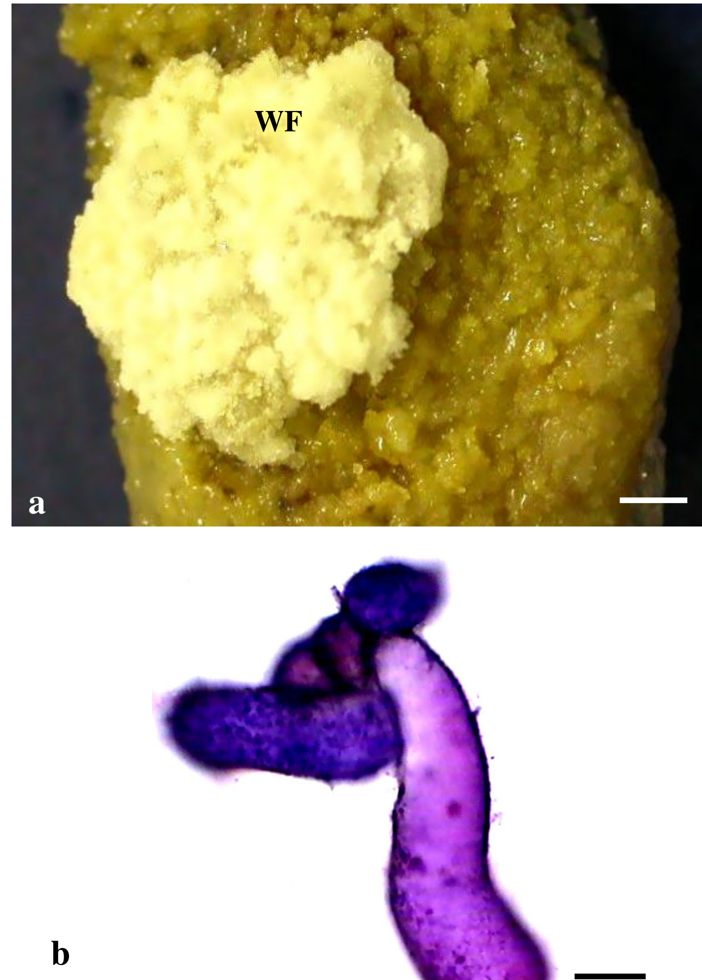


Figure 3.2: (a) White flaky (WF) callus present on embryonic axis segment exposed to medium containing naphthaleneacetic acid (NAA). Scale bar = 2 mm; (b) Non-embryogenic cells present in white flaky callus. Scale bar = 0.1 mm

Zygotic axis segments placed on MS medium containing 2,4-D (CIa-6 – CIa-13) reacted better than explants placed on NAA-containing medium (CIa-1 – CIa-5) in terms of both the percentage of explants forming callus and the type of callus formed (Table 3.9). Mathews (2004) also showed 2,4-D to be more conducive for callusing compared with NAA for *Bacopa monnieri* internodes and shoot tip explants. Three parameters are suggested to define the interaction involved in the induction of embryogenesis by auxin. These are: (1) auxin structure, (2) auxin concentration, and (3) time of exposure

(discussed in section 3.2.2.3.1 below). The auxin 2,4-D has been shown to be more potent than NAA in effecting differentiation in cell culture, which has been ascribed to its structure (Stuart and Strickland, 1989). Depending on the primary explant, certain auxins and the concentrations used appear to be superior in promoting embryogenesis compared with others. However, there are instances where primary explants respond equally well to both auxins, e.g. soybean cotyledon or radicle explants (Liu *et al*., 1992).

In the present study, low concentrations of 2,4-D (0.5 [CIa-6] and 0.7 mg l^{-1} [CIa-7]) resulted in 100% of explants producing white flaky as well as translucent callus (Figure 3.3). When placed on medium containing 3.0 mg l^{-1} 2,4-D (CIa-13) 70% of explants produced white flaky and translucent callus, which was not embryogenic (Table 3.9). The best results obtained were when embryonic axis segments were placed on MS medium incorporating 1.0 (CIa-8) or 2.0 mg l^{-1} (CIa-9) of 2,4-D which resulted in 86 and 100% of explants, respectively, producing callus (Table 3.9). These concentrations of 2,4-D resulted in the formation of yellow callus (Figure 3.4) which, when examined microscopically, revealed embryogenic cells identified by their large nuclei and isodiametric morphology (Figure 3.5). Yellow callus gave rise to globular structures, which formed after 3 - 4 weeks on the explant (Figure 3.6). Explants treated with 2.0 mg l^{-1} 2,4-D produced a greater number of globular structures per unit callus surface compared with those resulting when 1.0 mg l^{-1} 2,4-D was incorporated in the medium. Thus 2.0 mg l^{-1} 2,4-D was selected as the best concentration to use for subsequent experiments.

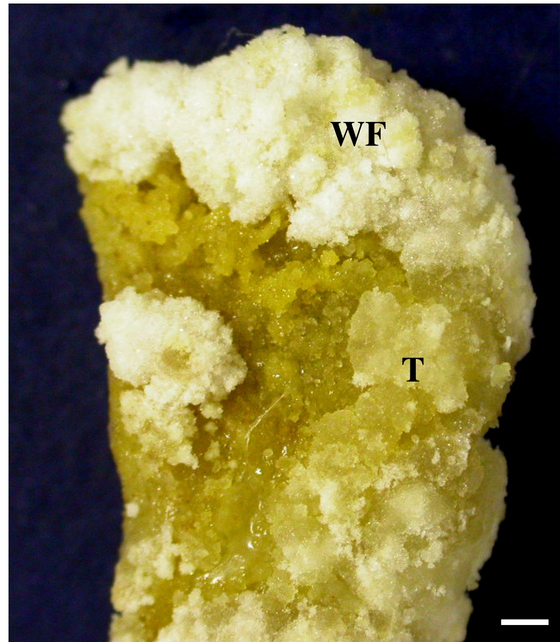


Figure 3.3: Different callus types formed on *B*. *racemosa* embryo explant. WF – White flaky callus, T – Translucent callus. Scale bar = 2 mm

Figure 3.4: Yellow callus formed on *B*. *racemosa* embryo explant. Scale bar = 3 mm

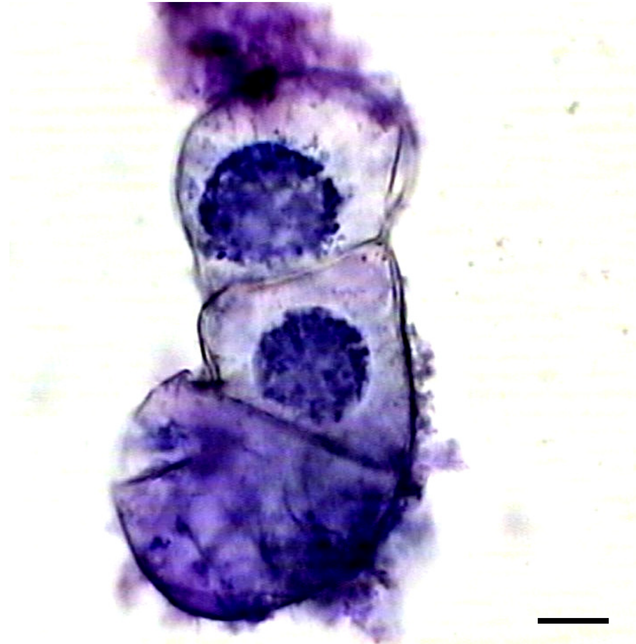


Figure 3.5: Embryogenic cells from yellow callus on *B. racemosa* explant. Scale bar = 0.1 mm

Figure 3.6: Globular structures (GS) forming from yellow callus on *B. racemosa* embryonic axis segment. Scale bar = 2 mm

3.2.4.1 The incorporation of cytokinins to the callus induction medium to enhance somatic embryo formation

The incorporation of cytokinins at low concentrations into culture media in the presence of an auxin has been used to stimulate somatic embryogenesis (George, 1996). Biswas *et al*. (2007) were able to produce large numbers of embryos when leaf and nodal segments of *Fragaria spp*. were placed on MS medium supplemented with 1.0 mg l^{-1} 2,4-D and 0.5 mg l^{-1} BAP. However, auxin/cytokinin combinations are not always efficacious: for example, addition of 2,4-D or NAA in combination with either BAP, TDZ or kinetin to MS medium suppressed the formation of embryos on zygotic axes and cotyledon explants of *Protea cynaroides* (Wu *et al*., 2007). The use of growth regulators for the induction of somatic embryos appears to be strongly species-as well as explant-specific.

In this study, zygotic embryo explants were exposed to 2.0 mg l^{-1} 2,4-D in combination with TDZ or BAP in attempts to enhance the formation of globular structures as well as to promote somatic embryo growth (Table 3.10). There was no significant difference in the percentage of explants producing callus whether TDZ or BAP was included in the induction media. However, globular structures did not form on embryo segments placed on medium supplemented with 2.0 mg l^{-1} 2,4-D in combination with 0.01 and 0.05 mg l^{-1} TDZ (CIb-1 and CIb-2, respectively) and 1.0 mg l^{-1} BAP (CIb-6).

Medium supplemented with 0.1 mg l^{-1} BAP (CIb-4) produced the most globular structures, which were defined and large compared with those forming on explants from the other treatments (Figure 3.7). White flaky and translucent calli were not embryogenic, and were fine and loose in nature as described above.

Table 3.10: The effect of 2.0 mg l^{-1} 2,4-D in combination with either TDZ or BAP in the induction medium. Embryo explants were plated on MS medium supplemented with 30 g l^{-1} sucrose. **Types of callus: WF -** White flaky**, T -** Translucent, **Y** - Yellow and **GS -** Globular structures. **Amount of callus scored as**: **(-)** = no callus, **(+) =** quarter of explant, **(++)** = half of explant, **(+++) =** three quarter of explant and **(++++)** = whole explant. n=50. Mean values represented by the same alphabetical letters are not significantly different (Chi-squared test, $p < 0.05$).

Medium Code	% explants producing callus	Types of callus	Amount Of Callus
CIb-1	90 [a]	T	++
		WF	+
CIb-2	100 [a]	T	++
		WF	++
CIb-3	100 [a]	Y	++
		GS	+
		WF	+
CIb-4	100 [a]	GS	++
		Y	+
		WF	+
CIb-5	100 [a]	Y	++
		GS	+
		WF	+
CIb-6	100 [a]	WF	++
		T	+

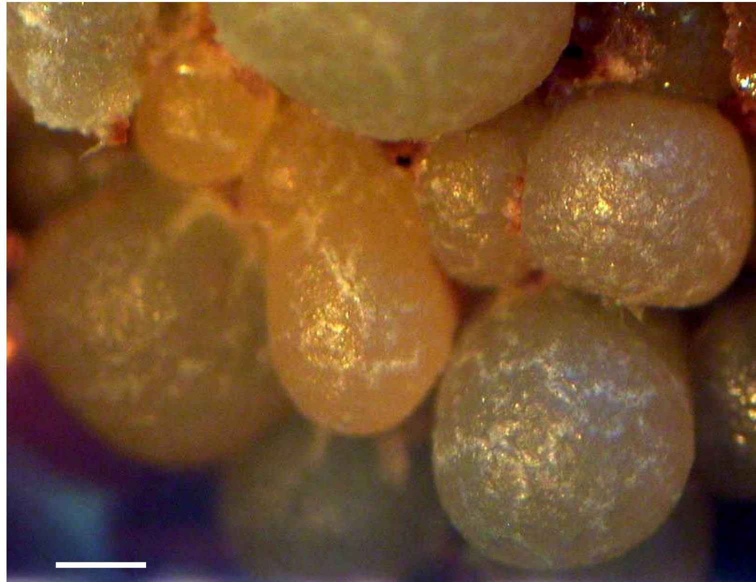


Figure 3.7: Large defined globular structures forming on embryo explants plated on MS medium supplemented with 2.0 mg l^{-1} 2,4-D and 0.1 mg l^{-1} BAP. Scale bar = 0.5 mm

3.2.4.2 Effect of liquid medium pre-treatment on somatic embryo induction

The use of liquid medium pre-treatment has been used in protocols to generate somatic embryos, e.g. for peach palm (Steinmacher, 2007), *Coffea arabica* (Quiroz-Figueroa *et al*., 2002) and *Oryza sativa* (Nhut *et al*., 2000). Liquid medium is easily accessible to the explant, which facilitates explants taking up the nutrients they require more efficiently.

In this study, embryo segments were placed in RITA vessels for different lengths of time and were flushed for 30 sec with medium every 30 min. *Barringtonia racemosa* axis segments that were pre-treated for 24 h and 3 d produced globular embryos when treated with growth-regulator-containing medium after the pre-treatment (Table 3.11). Explants plated on hormone-free MS medium did not produce any globular structures after the pre-treatment. There was no enhancement in the promotion of globular embryo development, compared with the previous study, using solid media only. There was also a positive correlation between contamination percentages and treatment period, therefore the effects of longer pre-treatment periods were not assessed. As the use of

liquid pre-treatment did not enhance the production of *B. racemosa* somatic embryos, and in view of the contamination, further use of the RITA system was not pursued.

Table 3.11: The effect of liquid medium containing 2.0 mg l^{-1} 2,4-D and 0.1 mg l^{-1} BAP on embryonic axis segments held in RITA vessels and flushed for 30 seconds every 30 minutes. After incubation in the RITA vessel explants were plated onto either MS medium containing (a) 2.0 mg l^{-1} 2,4-D and 0.1 mg l^{-1} BAP; or (b) devoid of PGR. **Types of callus: WF -** White Flaky, **T -** Translucent, **Y** - Yellow callus and **GS** - Globular structures. **Amount of callus scored as**: (-) = no callus, **(+) =** quarter of explant, **(++)** = half of explant, **(+++) =** three quarter of explant and **(++++)** = whole explant. n=20. Mean values represented by the same alphabetical letters within each column are not significantly different (Chi-squared test, $p < 0.05$).

Time	Medium following RITA vessel	% Explants producing callus	Types of callus	Amount Of Callus	Contamination (%)
24 h	a	100 [a]	T GS	++ +	0 [c]
	b	80 [a]	T WF	++ +	10 [c]
3days	a	100 [a]	GS Y WF	++ + +	60 [b]
	b	100 [a]	T WF	++ +	50 [b]
7 days	a	-	-	-	100 [a]
	b	-	-	-	100 [a]

3.2.4.3 Microscopical investigation of globular structures

Globular structures forming on *B. racemosa* axis segments were embedded in wax (Materials and Methods, section 3.1.4) to determine if these were, in fact, somatic embryos. If so, these structures would be suitable explants for cryopreservation since each was an isolated unit, which easily dislodged itself from the parental callus mass.

Globular structures when embedded sectioned and viewed with the light microscope revealed a surrounding layer of necrotic cells with dividing cells in the interior. The most notable aspect was the shape of section. Figure 3.8 (a) shows a typical heart shaped structure, which is one of the development stages in embryogenesis, and Figure 3.8 (b) shows further development of the structure and two definite poles, a possible shoot and root region that were darkly stained areas compared with the rest of the section. These structures, showing certain characteristics of somatic embryos, were considered to have the potential for germination.

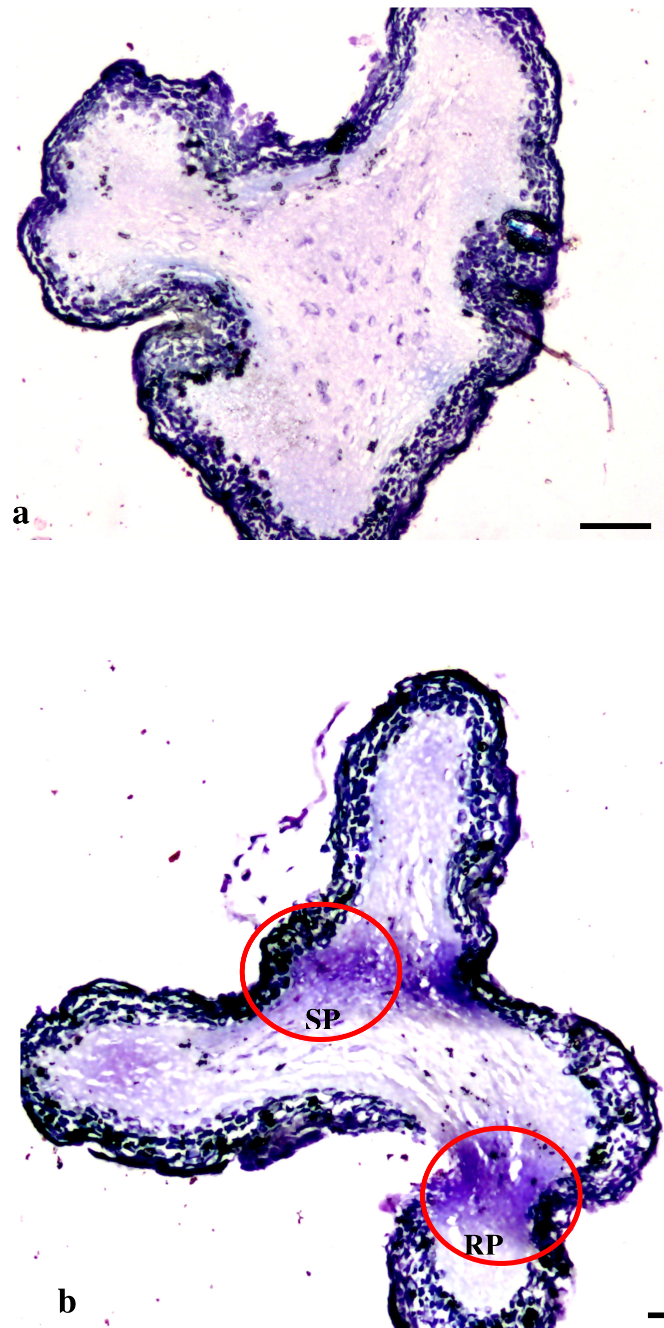


Figure 3.8: Light microscopy images of wax embedded globular structures illustrated by (a) heart shaped embryo and (b) more developed stage showing a distinctive shoot pole (SP) and root pole (RP). Scale bar = 0.1 mm

3.2.4.4 Effect of zygotic axis maturity on somatic embryo induction

The maturity of the primary explant plays an important role in the initiation of somatic embryos. Immature tissues are usually favoured since they are considered more plastic in nature compared with mature tissues. Berros *et al.* (1995) investigated the effects of seed maturity on somatic embryo induction for *Corylus* spp. (hazel nut), to ascertain whether macromorphological characterisation during seed development might be correlated with embryogenic ability. Juvenile zygotic axis tissue, identified in the context of the size of the fruit, had greater embryogenic capacity compared with more mature axis tissues.

Along these lines, in this study three size classes of *B. racemosa* fruit, which were differentiated according to mass, length and breadth as a measure of maturity, were investigated. Only green fruit were used since zygotic axes from red/brown fruit were either rotten or had been predated by insects.

After six weeks in culture *B. racemosa* zygotic axis segments produced globular and heart-shaped somatic embryos from all three sizes of zygotic axes tested, although there was a significant difference in the percentage of explants producing embryos (Table 3.12). All segments from class 3 (most mature) produced embryos, while 63 and 73% of explants produced somatic embryos on explants from classes 1 and 2, respectively. These results are interesting in that explants from the least mature zygotic embryos gave the poorest yield of globular embryos. However, it must be pointed out that in *B. racemosa*, the entire nutrient reserve of the seed is incorporated into the hypertrophied axis (Berjak *et al.*, 1996). Thus, it is suggested that the capability for somatic embryogenesis by axis tissues is increasingly enhanced as nutrients (and possibly other factors such as PGRs) are accumulated.

For subsequent experiments class 3 embryos were used since they had responded the best in culture.

Table 3.12: The effect of explant maturity on somatic embryo formation. **No. of embryos: (+)** = 1 - 5 embryos, **(++)** = 6 - 10 embryos, **(+++)** = 11 - 20 embryos, **(++++)** = > 20 embryos. n=30. Mean values represented by the same alphabetical letters are not significantly different (Chi-squared test, $p < 0.05$).

Embryo size	% explants producing embryos	Globular embryos	Heart shaped embryos
Class 1	63 [b]	++	+
Class 2	73 [b]	++	+
Class 3	100 [a]	+++	++

3.2.4.5 Effect of sucrose concentration on embryo formation

From studies on various species, the form and concentration of exogenous sugars included in the culture medium is suggested to affect somatic embryogenesis (Daigny *et al*., 1996; Agarwal *et al*., 2004). Sugars provide an energy and carbon source for explants, as well as acting as an osmoticum. Elevated sucrose concentration in culture media has been observed to promote osmotic activity of explants, and the osmotic stress created appears to improve embryogenesis (Agarwal *et al*., 2004). As examples, inclusion of 6% sucrose improved maturation of black and white spruce somatic embryos (Iraqi and Tremblay, 2001) and those of *Morus alba* (Agarwal *et al*., 2004).

In the present study, however, increasing the concentration of sucrose in the medium resulted in a decreasing number of explants producing embryos (Table 3.13). The callus formed was white and flaky and not embryogenic (results not shown). Sucrose concentrations higher than 30 g l^{-1} were not advantageous for the formation of *B. racemosa* somatic embryos since the ability of explants to produce embryos declined and further development of globular structures was not promoted. Therefore, for all subsequent studies the concentration of sucrose used was 30 g l^{-1}.

Table 3.13: The effect of sucrose on somatic embryo formation. **No. of embryos: (+)** = 1 - 5 embryos, **(++)** = 6 - 10 embryos, **(+++)** = 11 - 20 embryos, **(++++)** = > 20 embryos. n=15-20. Mean values represented by the same alphabetical letters are not significantly different (Chi-squared test, $p < 0.05$).

Concentration of sucrose (g l^{-1})	% Explants producing embryos	Globular embryos	Heart shaped embryos
30	84 [a]	+++	-
60	26 [bc]	+	-
90	44 [b]	+	-
120	16 [c]	+	-

3.2.4.6 Effect of casein hydrolysate on embryo formation

Casein hydrolysate (CH) provides many valuable nutrients that are essential for the formation and development of somatic embryos (George, 1996; Razdan, 2003; Steinmacher *et al*., 2007). In the present study, after six weeks in culture there was no significant difference in the percentage of explants producing embryos for any of the three CH concentrations tested (Table 3.14). However, explants placed on 1.0 g l^{-1} CH, the highest concentration used, produced heart shaped embryos (1-5 embryos) while explants placed on 0.1 and 0.5 g l^{-1} CH did not. For subsequent experiments 1.0 g l^{-1} CH was incorporated into the embryo induction medium.

Table 3.14: The effect of casein hydrolysate (CH) on somatic embryo formation. **No. of embryos: (+)** = 1 - 5 embryos, **(++)** = 6 - 10 embryos, **(+++)** = 11 - 20 embryos, **(++++)** = > 20 embryos. n=20-30. Mean values represented by the same alphabetical letters are not significantly different within columns (Chi-squared test, $p < 0.05$).

Concentration of CH (g l^{-1})	% Explants producing embryos	Globular embryos	Heart shaped embryos
0.1	70 [a]	++	-
0.5	75 [a]	+	-
1.0	90 [a]	++	+

3.2.5 Somatic embryo maturation

Embryo maturation and viable plantlet formation in many plant systems is frequently a challenging step when establishing a somatic embryogenesis protocol (Junaid *et al*., 2006). The progression of embryo development from early globular to globular, proceeding to the heart, torpedo and finally cotyledonary stage differs in requirements among species as well as among genotypes within the same species (Park *et al*., 2006; Steinmacher *et al*., 2007).

Various factors influence the maturation of early stage embryos: various types of growth regulators, levels of nitrogen, evolved gases, the concentration of carbohydrates: the timing of application of some the factors mentioned above seem to be important for somatic embryo maturation (Park and Son, 1995; Feher *et al*., 2003; Razdan, 2003).

3.2.5.1 Optimisation of explant exposure to auxin containing induction medium

The length of time explants are exposed to auxin-containing media is one of the main factors that influences embryo development (George, 1996; Razdan, 2003). Some species are able to produce all the consecutive stages of embryogenesis on a single medium composition. Other species, however, require the removal of the explant from the primary callus induction medium onto a secondary callus medium usually lacking auxin or containing a very low concentration of auxin, to facilitate embryo maturation and germination (George, 1996; Razdan, 2003). The establishment of an induction culture medium that promotes the formation of embryogenic cells, with subsequent transfer to a secondary medium that aids with embryo formation and maturation, i.e. a two-step procedure, is common practice across species for embryo formation (George, 1996). The removal of auxin from the culture medium is regarded as a prerequisite to 'switch off' several genes and to stimulate the synthesis of new gene products that are necessary for the successful completion of embryo development (Zimmerman, 1993).

To investigate the time for which explants of *B. racemosa* zygotic embryos should best be incubated on the induction medium (MS medium, 30 g l^{-1} sucrose, 2.0 mg l^{-1} 2,4-D, 0.1 mg l^{-1} BAP, 1.0 g l^{-1} CH and 8.0 g l^{-1} bacteriological agar), they were maintained for 1, 2, 3 and 4 weeks on auxin-containing medium. Thereafter explants were transferred

onto a secondary callus medium without auxin but otherwise modified in ways recorded to enhance embryo maturation.

3.2.5.1.1 The influence of reduced nitrogen supply in the secondary callus medium

A reduction in the strength of the basal medium was the first factor investigated, since a reduced nitrogen supply has been found to improve embryogenesis for e.g. *Santalum album* (Das *et al*., 2001) and for sorghum (Elkonin and Pakhomova, 2000). In the present study, after the explants were exposed to the callus induction medium, for 1 – 4 weeks, they were placed for a further 4 weeks onto secondary callus media S1 and S2, half-strength and full-strength MS medium respectively. Reducing the strength of the MS medium did not, however, have any promotional effect on the formation of somatic embryos of *B. racemosa*, irrespective of the incubation period on the induction medium (Table 3.15).

Zygotic axis segments incubated on the induction medium for two or three weeks with subsequent transfer onto the full-strength MS medium (S2) produced globular embryos. However, the omission of auxin from the secondary culture medium was not sufficient to enhance the further development of somatic embryos beyond the globular stage. In contrast, there are published accounts that the transfer of calli to a PGR-free medium was sufficient to induce embryo development to the cotyledonary stage for *Salvia officinalis* and *S. fruticosa* (Kintzios *et al*., 1999) and towards germination for *Lilium longiflorum* (Nhut *et al*., 2006).

Table 3.15: Zygotic axis segments cultured on the induction medium for 1 - 4 weeks, and then transferred onto auxin free media S1 and S2 for a further 4 weeks to promote embryo development. **S1** - half-strength MS, **S2** – full-strength MS. **Types of callus**: **WF** - White flaky, **T -** Translucent, **Y** - Yellow callus, **GS** - Globular structures. **Amount of callus scored as**: (-) = no callus, **(+) =** quarter of explant, **(++)** = half of explant, **(+++) =** three quarter of explant and **(++++)** = whole explant. n=20. Mean values represented by the same alphabetical letters are not significantly different within columns (Chi-squared test, $p < 0.05$).

Medium	No. of weeks on induction medium	% explants producing callus	Callus type	Amount of callus
S1	1	100 [a]	T	++
			WF	+
	2	80 [ab]	Y	++
			T	+
			WF	+
	3	100 [a]	WF T	+++
	4	80 [ab]	T	++
S2	1	80 [ab]	T WF	+++
	2	100 [a]	GS	++
			Y	++
	3	100[a]	GS	+++
			Y	+
	4	60 [b]	T	++

3.2.5.1.2 The influence of BAP in the secondary callus medium

The inclusion of BAP in the secondary callus medium has been shown to promote the development of somatic embryos for e.g. *Eucalyptus grandis* (Tsewana, 2001) and *Juglans regia* (Kaur *et al*., 2006). Cytokinins in general, promote cell division and regulate growth and development of plant tissues and organs (Razdan, 2003). In the present study, explants were removed from the auxin-containing induction medium after 1 - 4 weeks and placed for a further 4 weeks on full-strength MS medium containing 0.1

mg l^{-1} BAP (medium S3) or 1.0 mg l^{-1} BAP (medium S4). *Barringtonia racemosa* explants did not respond to either of the BAP concentrations incorporated into the secondary culture medium irrespective of the incubation duration on the induction medium (Table 3.16). Although BAP did have an influence on globular embryo formation when incorporated into the induction medium (see Table 3.10), there was no difference in the types of callus formed compared with the previous experiments.

Table 3.16: Zygotic axis segments incubated on the callus induction medium for 1 - 4 weeks then transferred onto media S3 and S4 for a further 4 weeks to promote somatic embryo development. **S3** - 0.1 mg l^{-1} BAP, **S4** – 1.0 mg l^{-1} BAP. **Types of callus**: **WF**- White flakes, **T-** Translucent, **Y**-Yellow callus. **Amount of callus scored as**: (-) = no callus, **(+) =** quarter of explant, **(++)** = half of explant, **(+++) =** three quarter of explant and **(++++)** = whole explant. n=20. Mean values represented by the same alphabetical letters are not significantly different within columns (Chi-squared test, $p < 0.05$).

Medium	No. of weeks on Induction medium	% explants producing callus	Callus type	Amount of callus
S3	1	60^{b}	Y	++
			WF	+
	2	80^{ab}	T	++
			WF	+
	3	100^{a}	T	++
			WF	+
	4	100^{a}	T	++
			WF	+
S4	1	60^{b}	T	+
			WF	+
	2	100^{a}	Y	++
			T	+
	3	100^{a}	T	++
			WF	++
	4	100^{a}	T	+++

3.2.5.1.3 The influence of ABA in the secondary callus medium

Abscisic acid (ABA) has been distinguished as an important growth regulator for *in vitro* somatic embryo maturation and an effective additive in the medium supporting growth of globular and early heart-shaped somatic embryos (Capuana and Debergh, 1997). Salajova *et al*. (1999) suggested that ABA at the histological level influences the internal organization of developing somatic embryos. This PGR has been implicated in the acquisition of desiccation tolerance and is suggested to induce the storage of reserves such as starch, sucrose, proteins and lipids in seeds (Torres *et al*., 2001). The addition of ABA to the secondary culture medium has been reported to result in embryo maturation for *Larix occidentalis* (Von Aderkas *et al*., 1995), *Psoralea corylifolia* (Sahrawat and Chand, 2001), *Pinus brutia* (Yildirim *et al*., 2006) and *Larix leptolepsis* (Kim and Moon, 2007).

In the present study, the inclusion of ABA into the secondary culture medium did not have any discernible effect on somatic embryo development. Explants incubated for 3 weeks on the induction medium with subsequent transfer for a further 4 weeks onto media containing either 0.1 mg l^{-1} ABA (S5) or 1.0 mg l^{-1} ABA (S6) produced globular embryos (Table 3.17). However, over 8 weeks in culture on the secondary callus medium no further development was recorded.

Table 3.17: Zygotic axis segments incubated on the callus induction medium for 1 - 4 weeks then transferred onto media S5 and S6 for a further 4 weeks to promote embryo development. **S5** - 0.1 mg l^{-1} ABA, **S6** – 1.0 mg l^{-1} ABA. **Types of callus**: **WF**- White flakes, **T-** Translucent, **Y**-Yellow callus, **GS**-Globular structures. **Amount of callus scored as**: (-) = no callus, **(+) =** quarter of explant, **(++)** = half of explant, **(+++) =** three quarter of explant and **(++++)** = whole explant. n=20. Mean values represented by the same alphabetical letters are not significantly different within columns (Chi-squared test, $p < 0.05$).

Medium	No. of weeks on Induction medium	% explants producing callus	Callus type	Amount of callus
S5	1	100 [a]	T	+++
	2	100 [a]	T	+++
	3	100 [a]	T WF GS	++ + +
	4	100 [a]	T WF	++ +
S6	1	100 [a]	T	++
	2	80 [a]	T WF	++ +
	3	100 [a]	Y GS	++ +
	4	50 [b]	T WF	+ ++

The most favourable incubation time on the induction medium was 3 weeks (see Tables 3.15 and 3.17), explants exposed for shorter periods not producing globular embryos. When explants were removed from the induction medium after four weeks and subsequently placed on the secondary callus medium, in all cases they appeared cracked and did not produce any globular embryos (ref. Tables 3.15, 3.16 and 3.17).

In an attempt to observe the effects of longer incubation periods, embryonic axis segments were incubated for either six or eight weeks on the induction medium and

thereafter placed on full-strength MS medium for a further 4 weeks. All the zygotic axis segments placed for six weeks on the induction medium produced globular embryos (results not shown). However, after eight weeks a white flaky callus began to proliferate over the globular structures, as well as the under-surface of the explant.

3.2.5.2 The effects of three different auxins at low concentrations used singly with the addition of either BAP or kinetin, on embryo maturation.

For these experiments, explants were incubated for six weeks on the induction medium and the effects of three auxins, IAA, 2,4-D and NAA used singly at low concentrations in combination with two cytokinins, BAP or kinetin, were investigated for explants on the secondary callus medium. According to Zimmerman (1993) once embryogenesis is induced, embryos begin to synthesise their own auxins thus requiring a lower exogenous auxin concentration. However, at this stage provision of a cytokinin maybe necessary for embryo maturation. For example, the transfer of *Psophocarpus tetragonolobus* callus cultures from NAA- and BAP-containing induction medium to a secondary medium incorporating 0.1 mg l^{-1} IAA and 0.2 mg l^{-1} BAP, resulted in 60% somatic embryo germination (Ahmed *et al*., 1995). In the present study, however, none of the combinations of growth regulators tested at any of the concentrations used, promoted development of globular stage somatic embryos towards germination (Tables 3.18, 3.19 and 3.20). The inclusion of IAA or NAA in the secondary culture medium, however, produced a higher frequency of heart shaped embryos compared with 2,4-D.

A significantly higher proportion of explants produced embryos when 0.7 mg l^{-1} IAA was combined with 1.0 mg l^{-1} kinetin, than any other combination of these PGRs (Table 3.18). In contrast, the lowest percentage of explants producing embryos was on medium supplemented with 0.1 mg l^{-1} IAA and 1.0 mg l^{-1} kinetin, implying, that this concentration of kinetin alone, was not the factor, promoting embryo formation and development. It appeared that the higher concentration of IAA (0.7 mg l^{-1}) was the major promoting factor. Although not significantly different, the combination of 0.1 mg l^{-1} BAP with 0.7 mg l^{-1} IAA resulted in a reduced percentage of explants producing globular embryos and there was no formation of heart shaped embryos compared with all other treatments where 0.7 mg l^{-1} IAA was provided.

Table 3.18: The effect of IAA in combination with BAP and kinetin on embryo maturation. Explants were incubated on the callus induction medium for six weeks thereafter incubated on the secondary callus medium for a further six weeks. **No. of embryos:** **(+)** = 1 - 5 embryos, **(++)** = 6 - 10 embryos, **(+++)** = 11 - 20 embryos, **(++++)** = > 20 embryos. n=15. Mean values represented by the same alphabetical letters are not significantly different within columns (Chi-squared test, $p < 0.05$).

Growth regulator concentration (mg l^{-1})			Explants producing embryos (%)	Globular embryos	Heart shaped embryos
IAA	BAP	KN			
0.1	0.1	-	40 [ab]	++	-
0.1	1.0	-	53 [ab]	++	+
0.1	-	0.1	40 [ab]	++	+
0.1	-	1.0	20 [b]	+	-
0.7	0.1	-	33 [ab]	+	-
0.7	1.0	-	53 [ab]	++	+
0.7	-	0.1	46 [ab]	+	+
0.7	-	1.0	60 [a]	+++	+

There was no significant difference in the percentage of explants producing embryos with the provision of NAA in combination with BAP or kinetin to the secondary culture medium (Table 3.19). Explants placed on medium containing 0.1 mg l^{-1} NAA produced more globular embryos compared with treatments supplemented with 0.7 mg l^{-1} NAA and there was a higher frequency of explants where heart-shaped embryos had formed.

Table 3.19: The effect of NAA in combination with BAP and kinetin on embryo maturation. Explants were incubated on the callus induction medium for six weeks thereafter incubated on the secondary callus medium for a further six weeks. **No. of embryos:** **(+)** = 1 - 5 embryos, **(++)** = 6 - 10 embryos, **(+++)** = 11 - 20 embryos, **(++++)** = > 20 embryos. n=15. Mean values represented by the same alphabetical letters are not significantly different within columns (Chi-squared test, $p < 0.05$).

Growth regulator concentration (mg l^{-1})			Explants producing embryos (%)	Globular embryos	Heart shaped embryos
NAA	BAP	KN			
0.1	0.1	-	40 [a]	++	-
0.1	1.0	-	46 [a]	++	+
0.1	-	0.1	53 [a]	++	+
0.1	-	1.0	53 [a]	++	+
0.7	0.1	-	33 [a]	+	-
0.7	1.0	-	46 [a]	+	+
0.7	-	0.1	33 [a]	+	-
0.7	-	1.0	26 [a]	+	-

There was a significant difference in the percentage of explants producing embryos when placed on secondary culture medium supplemented with 2,4-D in combination BAP or kinetin (Table 3.20). Significantly lower percentages of explants produced embryos on media supplemented with both 0.1 and 0.7 mg l^{-1} 2,4-D in combination with 1.0 mg l^{-1} kinetin. There was no real pattern in the behaviour of explants exposed to the different concentrations of growth regulators, but the incorporation of 0.1 mg l^{-1} 2,4-D in combination with 0.1 mg l^{-1} kinetin produced the significantly highest percentage of explants with embryos while the addition of 0.1 mg l^{-1} kinetin to the culture medium promoted development of some heart-shaped embryos irrespective of the concentration of 2,4-D incorporated.

Table 3.20: The effect of 2,4-D in combination with BAP and kinetin on embryo maturation. Explants were incubated on the callus induction medium for six weeks thereafter incubated on the secondary callus medium for a further six weeks. **No. of embryos:** **(+)** = 1 - 5 embryos, **(++)** = 6 - 10 embryos, **(+++)** = 11 - 20 embryos, **(++++)** = > 20 embryos. Mean values represented by the same alphabetical letters are not significantly different within columns (Chi-squared test, $p < 0.05$).

Growth regulator concentration (mg l^{-1})			Explants producing embryos (%)	Globular embryos	Heart shaped embryos
2,4-D	BAP	KN			
0.1	0.1	-	26 ^{b}	+	-
0.1	1.0	-	33 ^{ab}	+	-
0.1	-	0.1	66 ^{a}	++	+
0.1	-	1.0	26 ^{b}	+	-
0.7	0.1	-	53 ^{ab}	++	+
0.7	1.0	-	40 ^{ab}	+	-
0.7	-	0.1	26 ^{b}	+	+
0.7	-	1.0	20 ^{b}	+	-

It was concluded that, in the case of *B. racemosa* zygotic axis-derived explants, the use of a secondary callus medium with the addition of the various combinations of growth regulators tested was not beneficial in the further development of globular stage embryos. In some cases, globular embryos produced proceeded to the heart stage of development, but no further development was recorded.

3.2.5.3 Desiccation of globular structures

The use of desiccation to promote embryo development has been used for *Picea rubens* (Harry and Thorpe, 1991); *Cynodon dactylon* (Ramgareeb, 1997), *Eucalyptus grandis* (Tsewana, 2001), *Pinus radiata* (Klimaszewska and Cyr, 2002), English walnut (Tang *et al*., 2000) and several other species. This procedure mimics seed maturation *in vivo,* and may be necessary to trigger metabolic processes needed for germination (Razdan, 2003). *Barringtonia racemosa* zygotic axis explants having formed globular stage

embryos within embryogenic callus, were desiccated for various periods and subsequently regenerated on MS medium devoid of plant growth regulators (Table 3.21). Although there was no significant difference in the percentage of explants producing embryos, embryogenesis was stimulated in explants desiccated for 24 h between two sheets of sterile filter paper, additional globular embryos being produced with further development to the heart and torpedo stages being recorded (Fig. 3.9). The 3 h desiccation period appeared to be too short to stimulate onwards development of globular embryos. Generally, however, desiccation of explants appeared to promote the development of *B. racemosa* somatic embryos.

Table 3.21: The effect of desiccation on embryo maturation. Explants were incubated for six weeks on the induction medium, desiccated and subsequently transferred onto MS medium devoid of PGRs for a further six weeks. **No. of embryos: (+)** = 1 - 5 embryos, **(++)** = 6 - 10 embryos, **(+++)** = 11 - 20 embryos, **(++++)** = > 20 embryos. n=15. Mean values represented by the same alphabetical letters are not significantly different within columns (Chi-squared test, $p < 0.05$).

Desiccation period	Explants producing embryos (%)	Globular embryos	Heart shaped embryos	Torpedo shaped embryos
3 h	66 [a]	++	-	-
6 h	53 [a]	++	+	-
12 h	66 [a]	++	+	-
24 h	80 [a]	+++	++	+

3.2.5.4 Incubation of explants at low temperature

The incubation of explants producing globular stage embryos at low temperatures in the range of 4 - 10˚C with and without the influence of desiccation has been recorded as beneficial in promoting embryo development and germination for red spruce (Harry and Thorpe,1991), Panax ginseng (Choi *et al*., 1999), *Juglans regia* (Tang *et al*., 2000), *Picea glauca* (Pond *et al*., 2002) and *Gossypium hirsutum* (Chaudhary *et al*., 2003). Usually species that require cold treatment for seed germination also require the application of chilling for somatic embryo germination (Razdan, 2003). However, with this method the chilling sensitivity of the tissue has to be taken into account to ensure that the material is not damaged or the regenerative ability lost. In the present study, *B. racemosa* zygotic axes were not cold-treated to enhance seed germination but this method was investigated for the development and germination of somatic embryos since it has been broadly implicated for a variety of species.

The incubation of *B. racemosa* zygotic axis segments at 4˚C on MS medium devoid of PGRs for various incubation periods was beneficial (Table 3.22) and, after an incubation period of 8 h at 4˚C cotyledonary stage embryos were observed (Figure 3.10), although the proportion of explants for which embryogenesis was promoted was sufficiently lower than after the 4 h cold treatment. Longer incubation periods did not result in the formation of cotyledonary stage embryos probably because *B. racemosa*, being a tropical/sub-tropical species, is generally chilling sensitive. The impact of the combination of desiccation and cold treatment was not applied to *B. racemosa* axis segments because of time constraints, and the fruiting season being over.

Figure 3.9: The different stages of embryogenesis achieved for *B. racemosa* after desiccation. Scale bar = 3 mm

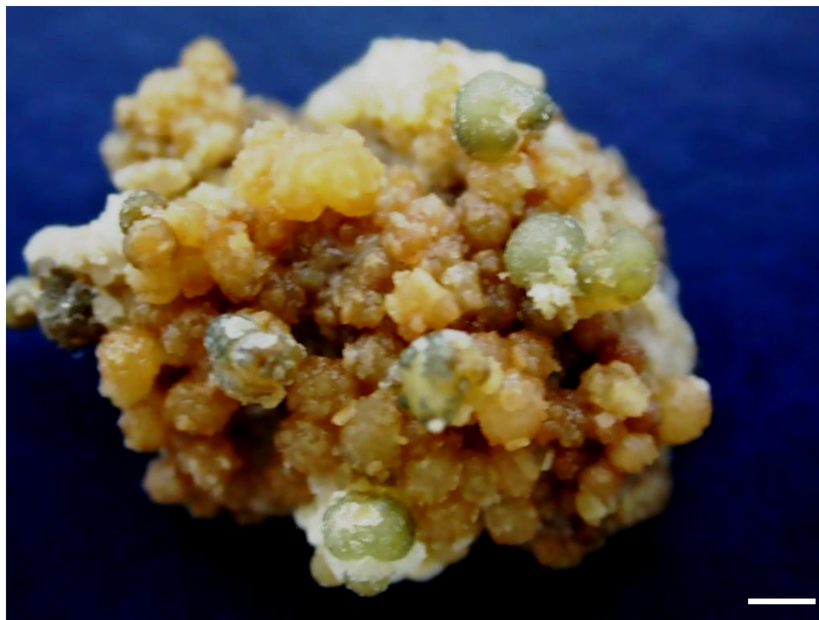


Figure 3.10: Cotyledonary stage somatic embryos forming after 8 h incubation at 4˚C. Scale bar = 3mm

Table 3.22: The effect of cold treatment on embryo maturation. Explants were incubated for 6 weeks on the callus induction medium thereafter transferred to MS medium devoid of PGRs, cold treated and subsequently stored in the dark at 25˚C for a further six weeks. **No. of embryos: (+)** = 1 – 5 embryos, **(++)** = 6 – 10 embryos, **(+++)** = 11 – 20 embryos, **(++++)** = > 20 embryos. N=15. Mean values represented by the same alphabetical letters were not significantly different within columns (Chi-squared test, $p < 0.05$).

Cold treatment duration	% explants producing embryos	Globular embryos	Heart shaped embryos	Cotyledonary embryos
4 h	86 [a]	+++	+	-
8 h	53 [b]	++	+	+
24 h	33 [b]	+	++	-
48 h	60 [ab]	++	+	-
96 h	60 [ab]	++	-	-
168 h	40 [b]	++	-	-

3.3 Conclusion

In vitro multiplication of shoots via embryonic axis explants was achieved. However, these explants were considered to be too large and thick for cooling in liquid nitrogen (for various reasons see Chapter 2 section, 2.2.2).

Attempts to generate somatic embryos did yield some success. A protocol (Figure 3.11) for globular stage embryo induction and development to the cotyledonary stage was achieved using 2-mm-thick embryonic axis segments as the primary explant. Both desiccation and cold treatment of globular stage embryos encouraged further development into the torpedo and cotyledonary stages of growth. This was achieved when explants were incubated for 8 h at 4˚C.

Although, embryo germination was not presently achieved, substantial progress towards somatic embryo formation has been made for *B. racemosa*, which could have major

implications for conservation of this recalcitrant-seeded species and others that are closely related.

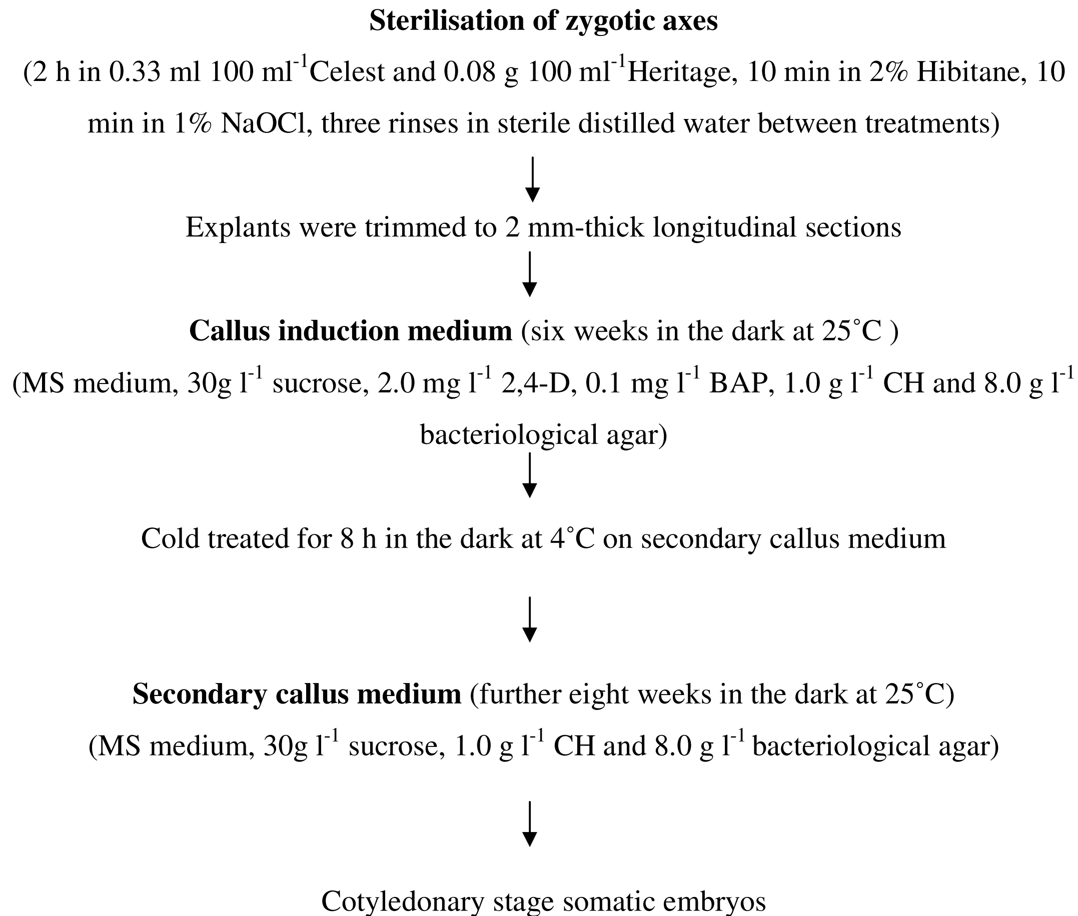


Figure 3.11: Protocol established for the production of cotyledonary stage somatic embryos from *B. racemosa* zygotic axis explants.

CHAPTER 4: GENERAL OVERVIEW AND FUTURE RESEARCH PROPOSALS

Conservation of plant genetic resources, especially woody tropical/subtropical species has been noted to be challenging (Berjak and Pammenter, 2007). Both species investigated in the present study, *T. cacao* and *B. racemosa*, belong to this selected category of species. In addition, both species of interest produce large seeds and zygotic axes that are not suitable as explants for cryopreservation.

Investigations toward developing cryopreservation protocols for the long-term conservation of both species using explants alternative to seeds and zygotic axes were carried out. The most important factor noted was that the choice of explant is crucial to success after cooling. Attempts to use axillary buds and *in vitro* nodal segments for *T. cacao* proved to be unsuccessful. Axillary buds lost regeneration ability if excised at the small size required for cooling while *in vitro* nodal segments proved to be extremely desiccation sensitive and none survived immersion into cryogen. This result re-iterates that survival after cryopreservation, especially for this group of species (woody, recalcitrant and tropical), is indeed challenging and other avenues or explants need to be investigated.

Barringtonia racemosa shoots that formed from zygotic axes were not suitable for cryopreservation due to their thickness and large size; however, they could have the potential to be used as material for slow growth cultures, affording medium-term storage of *B. racemosa* germplasm. Although somatic embryo germination was not achieved (largely due to time constraints and seasonality of seeds) for *B. racemosa*, the production of globular, heart, torpedo and cotyledonary stage somatic embryos was successful. Generally, the formation of somatic embryos from woody species is considered difficult (Wann, 1986) therefore the success obtained with *B. racemosa* is significant.

The use of meristems as explants for cryopreservation is suggested to be the way forward for the cryopreservation of both *T. cacao* and *B. racemosa*. These explants are

sufficiently small and contain meristematic cells that have the potential and vigour to regenerate when excised from the parental tissue. Recent success using meristems of *Trichilia emetica* and *T. dregeana* was achieved when explants were cryoprotected and cooled in liquid nitrogen (Varghese *et al*., 2008). *Trichilia* species, like *T. cacao* and *B. racemosa*, belong to the woody, recalcitrant and tropical/sub-tropical category of plants. Therefore that success holds much promise for the conservation of both species that have been the focus of the present study.

5. REFERENCES

Abdelnour-Esquivel, A. and Engelmann, F. (2002) Cryopreservation of Chayote (*Sechium edule* JACQ. SW.) zygotic embryos and shoot-tips from *in vitro* plantlets. *CryoLetters* **23**, pp. 299-308.

Adkins, S.W., Kunanuvatchaidach, R., Gray, S.J and Adkins, A.L. (1993) Effect of Ethylene and Culture Environment on Rice Callus Proliferation. *Journal of Experimental Botany* **44**(12), pp. 1829-1835.

Adu-Amponah, Y., Novak, F.G., Afza, R. and Van Duren, M. (1992) loc. cit. Traore, A., Maximova, S.N. and Guiltinan, M.J. (2003) Micropropagation of *Theobroma cacao* L. using somatic embryo-derived plants. *In Vitro Cellular and Developmental Biology* **39**, pp. 332-337.

Adu-Ampomah, Y., Novak, F.G., Afza, R., Van Duren, M. and Perea Dallos, M. (1988) loc. cit. Pence, V.C. (1995) Somatic embryogenesis in Cacao (*Theobroma cacao).* In: Y.P.S. Bajaj (Ed.) *Biotechnology in Agriculture and Forestry*, Vol. 30, *Somatic Embryogenesis and Synthetic Seed I*, Springer-Verlag, Berlin Heidelberg, pp. 455-467.

Afreen, F., Zobayed, S.M.A. and Kozai, T. (2002) Photoautotrophic Culture of *Coffea arabusta* Somatic Embryos: Development of a Bioreactor for Large-scale Plantlet Conversion from Cotyledonary Embryos. *Annals of Botany* **90**, pp. 21-29.

Agarwal, S., Kanwarand, K. and Sharma, D.R. (2004) Factors affecting secondary somatic embryogenesis and embryo maturation in *Morus alba* L. *Scientia Horticulturae* **102** (3), pp. 359-368.

Aguilar, M.E., Villalobos, V.M. and Vasquez, N. (1992) Production of cocoa plant (*Theobroma cacao* L.) via micrografting of somatic embryos. *In Vitro Cellular and Developmental Biology* **28**, pp. 15-19.

Ahmed, R., Gupta, S.D. and De, D.N. (1995) Somatic embryogenesis and plant regeneration from leaf derived callus of winged bean [*Psophocarpus tetragonolobus* (L.) DC.]. *Plant Cell Reports* **15**, pp. 531-535.

Anonymous (2002) Chocolate and its environment. Field Museum, pp. 7-10. Online: www.fieldmuseum.org/chocolate/education_pdf/choc_environ_reference.pdf.

Anonymous (2005) *Barringtonia racemosa*. Online:http://www.bihrmann.com/caudiciforms/subs/bar-rac-sub.asp.

Anonymous (2007) California department of pesticide regulation, Public report, Chlorhexidinegluconate.Online:http://www.cdpr.ca.gov/docs/registration/ais/publicreports/5933.pdf.

Anonymous (2008) Calcium hypochlorite. *Wikipedia, The Free Encyclopedia*. Online: http://en.wikipedia.org/w/index.php?title=Calcium_hypochlorite&oldid=219319274.

Ascough, G.D., Erwin, J. and Van Staden, J. (2007) In vitro propagation of four *Watsonia* species. *Plant Cell Tissue and Organ Culture* **88**, pp. 135-145.

Baek, H., Kim, H., Cho, E., Chae, Y. and Engelmann, F. (2003) Importance of explant size and origin and of preconditioning treatments for cryopreservation of garlic shoot apices by vitrification. *CryoLetters* **24**, pp. 381-388.

Bajaj, Y.P.S. (1995) Biotechnology in Agricultre and Forestry 32: *Cryopreservation of Plant Germplasm* 1. Springer, Berlin, pp. 512.

Bartlett, D.W., Clough, J.M., Godfrey, C.R.A., Godwin, G.R., Hall, A.A., Heaney, S.P. and Maund, S.J. (2001) Understanding the strobilurin fungicides. *Pesticide outlook* **8**, pp. 143-148.

Benson, E.E. and Bremner, D.H. (2004) Oxidative stress in the frozen plant: a free radical point of view. In: B. Fuller, N. Lane and E.E. Benson (Eds.) *Life in The Frozen State*, CRC Press, London, pp. 205–242.

Benson, E.E. and Withers, L.A. (1987) Gas chromatographic analysis of volatile hydrocarbon production by cryopreserved plant tissue cultures: a non-destructive method for assessing stability. *Cryoletters* **8**. pp. 35-46.

Benson, E.E. and Withers, L.A. (1988) The Application of Germplasm Storage in Biotechnology. In: M. Salomé S. Pais, F. Mavituna and J.M. Novais (Eds.), *Plant Cell Biotechnology*. Springer-Verlag, Berlin Heidelberg, pp. 431-443.

Berjak, P. and Dumet, D. (1996) Cryopreservation of seeds and isolated embryonic axes of neem *(Azadirachta indica)*. *Cryoletters* **17**, pp. 99-104.

Berjak, P., Farrant, J.M., Mycock, D.J. and Pammenter, N.W. (1990) Recalcitrant (homoiohydrous) seeds: the enigma of their desiccation sensitivity. *Seed Science and Technology* **18**, pp. 297- 310.

Berjak, P., Farrant, J.M. and Pammenter, N.W. (1989) The basis of recalcitrant seed behaviour. Cell biology of the homoiohydrous seed condition. In: R.B. Taylorson (Ed.) *Recent Advances in the Development and Germination of Seeds*. Plenum Press, New york, pp. 89-108.

Berjak, P., Kioko, J.I., Walker, M., Mycock, D.J., Wesley-Smith, J., Watt, P. and Pammenter, N.W. (1998) Cryopreservation-An elusive goal. In: M. Marzalina, K.C. Khoo, N. Jayanthi, F.Y. Tsan and B. Krishnapillay (Eds.) *Recalcitrant Seeds*. Proceedings of IUFRO Seed Symposium, Kuala Lumpur, Malaysia, pp. 96-109.

Berjak, P., Mycock, D.J., Wesley-Smith, J., Dumet, D. and Watt, M.P. (1996) Strategies for *in vitro* conservation of hydrated germplasm. In: M.N. Norma, M.K. Narimah and

M.M. Clyde (Eds) *In vitro conservation of plant genetic resources. Kuala Lumpur, Malaysia,* Percetakan Watan Sdn.Bhd, pp.19-52.

Berjak, P. and Pammenter, N.W. (2004) Recalcitrant seeds. In: R.L. Benech-Arnold and R. A. Sánchez (Eds.) *Seed Physiology*, *Application to Agriculture.* Harworth Press Inc., New York, pp. 305-345.

Berjak, P. and Pammenter, N.W. (2007) From *Avicennia* to *Zizania*: Seed Recalcitrance in Perspective. *Annals of Botany*, doi:10.1093/aob/mcm168.

Berjak, P., Walker, M., Watt, M.P. and Mycock, D.J. (1999) Experimental parameters underlying failure or success in plant germplasm cryopreservation: A case study on zygotic axes of *Querces robur* L. *CryoLetters* **20**, pp. 251-262.

Berros, B., Rey, M., Díaz-Sala, C., and Albuerne, M. (1995) Somatic embryogenesis in Hazel nut (*Corylus* Species). In: Y.P.S. Bajaj (Ed) *Somatic Embryogenesis and Synthetic Seed I, Biotechnology in Agriculture and Forestry* 30, Springer-Verlag Berlin Heidelburg, pp. 378-387.

Bhojwani, S.S. (1990) Plant Tissue Culture: Applications and Limitations, Elsevier, Amsterdam, pp. 1-461.

Biswas, M.K., Islam, R. and Hossain, M. (2007) Somatic embryogenesis in strawberry (*Fragaria* sp.) through callus culture. *Plant Cell Tissue and Organ Culture* **90**, pp. 49-54.

Blakesley, D. and Kiernan, R.J. (2001) Cryopreservation of axillary buds of a *Eucalyptus grandis* X *Eucalyptus camaldulensis* hybrid. *CryoLetters* **22**, pp. 13-18.

Bowers, J.H., Bailey, B.A., Hebbar, P.K., Sanogo, S. and Lumsden, R.D. (2001) The impact of plant diseases on world chocolate production. Online: Plant Health Progress doi:10.1094/PHP-2001-0709-01-RV.

Bruno, A., Marcotrigiano, M. and Horton, N.J. (2007) A three-stage micropropagation system for a novel color morph of the wildflower, *Porteranthus trifoliatus* (L.) Britt. *Plant Cell Tissue and Organ Culture* **90**, pp. 9-13.

Burke, M.J., Gusta, L.V., Quamme, H.A**.,** Weiser, C.J. and Li, P.H. (1976) Freezing and injury in plants. *Annual Review of Plant Physiology* **27**, pp. 507-528.

Capuana, M. and Debergh, P.C. (1997) Improvement of the maturation and germination of horse chestnut somatic embryos. *Plant Cell Tissue and Organ Culture* **48**, pp. 23-29.

Carman, J.G. (1990) Embryogenic cells in plant tissue cultures: Occurrence and behaviour. *In Vitro Cell and Developmental Biology* **26**, pp. 746-753.

Cella, R., Parisi, B. and Nielsen, E. (1982) Characterization of a carrot cell line resistant to azetidin-2-carboxylic acid. *Plant Science Letters* **24**, pp. 125-135.

Chaudhury, R. and Chandel, K.P.S. (1991) Cryopreservation of desiccated seeds of neem (*Azadirachta indica*) for germplasm conservation. *Indian Journal of Plant Genetic Resources* **4**, pp. 67–72.

Chaudhary, B., Kumar, S., Prasad, K.V.S.K., Oinam, G.S., Burma, P.K. and Pental, D. (2003) Slow desiccation leads to high-frequency shoot recovery from transformed somatic embryos of cotton (*Gossypium hirsutum* L. cv. Coker 310 FR). *Plant Cell Reports* **21**, pp. 955–960.

Charoensub, R., Hirai, D. and Sakai, A. (2004) Cryopreservation of *in vitro*-grown shoot tips of cassava by encapsulation-vitrification method. *CryoLetters* **25**, pp. 51-58.

Charoensub, R., Phansiri, S., Sakai, A. and Yongmanitchai, W. (1999) Cryopreservation of cassava in vitro-grown shoot tips cooled to -196°C by vitrification. *CryoLetters* **20**, pp. 89-94.

Chatelet, P. and Dufour, M. (1990) loc. cit. Pence, V.C. (1995) Somatic embryogenesis in Cacao (*Theobroma cacao)*. In: Y.P.S. Bajaj (Ed.) *Biotechnology in Agriculture and Forestry*, Vol. 30, *Somatic Embryogenesis and Synthetic Seed I*, Springer-Verlag, Berlin Heidelberg, pp. 455-467.

Chatelet, P., Michauxferriere, N. and Dublin, P. (1992) loc. cit. Pence, V.C. (1995) Somatic embryogenesis in Cacao (*Theobroma cacao)*. In: Y.P.S. Bajaj (Ed.) *Biotechnology in Agriculture and Forestry*, Vol. 30, *Somatic Embryogenesis and Synthetic Seed I*, Springer-Verlag, Berlin Heidelberg, pp. 455-467.

Chen, L., Wang, Y., Xu, C., Zhao, M. and Wu, J. (2006) *In vitro* propagation of *Lychnis senno* Siebold et Zucc., a rare plant with potential ornamental value. *Scientia Horticulturae* **107**, pp. 183-186.

Chin, H.F. and Roberts, E.H. (1980) Recalcitrant crop seeds. Tropical Press SDN. BHD., Kuala Lumpur.

Choi, Y.E., Yang, D.C., Yoon, E.S. and Choi, K.T. (1999) High-efficiency plant production via direct somatic single embryogenesis from preplasmolysed cotyledons of Panax ginseng and possible dormancy of somatic embryos. *Plant Cell Reports* **18**, pp. 493-499.

Clavero-Ramírez, I., Gálveez-Farfán, J., López-Aranda, J.M. and González-Benito, M.E. (2005) Apex cryopreservation of several strawberry genotypes by two encapsulation-dehydration methods. *CryoLetters* **26 (1)**, pp. 17-24.

Corredoira, E., San-José, M.C., Ballester, A. and Vieitez, A.M. (2004) Cryopreservation of zygotic embryo axes and somatic embryos of European chestnut. *CryoLetters* **25**, pp. 33-42.

Daigny, G., Paul, H., Sangwan, R.S. and Sangwan-Narreel, B.S. (1996) Factors influencing secondary somatic embryogenesis in *Malus x domestica* Borkh. *Plant Cell Reports* **16**, pp. 153-157.

Das, S., Ray, S., Dey, S. and Dasgupta, S. (2001) Optimisation of sucrose, inorganic nitrogen and abscisic acid levels for *Santalum album* L. somatic embryo production in suspension culture. *Process Biochemistry* **37**, pp. 51-56.

Debergh, P., Roggemans, J. and Standaert-De Metsenaere, R. (1990) Tissue culture in relation to ornamental plants. In: S.S. Bhojwani (Ed.) *Plant Tissue Culture: Applications and Limitations*, Elsevier Science Publishers B.V., Netherlands, pp. 161-189.

Debnath, S.C. (2007) A two-step procedure for *in vitro* multiplication of cloudberry (*Rubus chamaemorus* L.) shoots using bioreactor. *Plant Cell Tissue and Organ Culture* **88**, pp. 185-191.

Deraniyagala, S.A., Ratnasooriya, W.D. and Goonasekara, C.L. (2002) Antinociceptive effect and toxicological study of the aqueous bark extract of *Barringtonia racemosa* on rats. *Journal of Ethnopharmacology* **86**, pp. 21-26.

Dhooghe, E. and Van Labeke, M.C. (2007) *In vitro* propagation of *Helleborus* species. *Plant Cell Tissue and Organ Culture* **91**, pp. 175-177.

Dodds, J.H. (1991) *In vitro* methods for conservation of Plant Genetic Resources. Chapman and Hall, London.

Dos Santos, A.V.P., Cutter, E.G. and Davey, M.R. (1983) Origin and development of somatic embryos in *Medicago sativa* L. (alfalfa). *Protoplasma* **117**, pp. 107-115.

Dos Santos, A.V.P., Machado, R.D. (1989) A scanning electron microscope study of *Theobroma cacao* somatic embryogenesis. *Annals of Botany* **64**, pp. 293-296.

Driver, J.A. and Kuniyuki, A.H. (1984) *In vitro* propagation of Paradox walnut rootstock. *Horticultural Science* **19**, pp. 507-509.

Duan, X., Wu, G. and Jiang, Y. (2007) Evaluation of the antioxidant properties of Litchi fruit phenolics in relation to pericarp browning prevention. *Molecules* **12**, pp. 759-771.

Duhem, K., Le Mecier, N. and Boxus, P. (1989) loc. cit. Pence, V.C. (1995) Somatic embryogenesis in Cacao (*Theobroma cacao)*. In: Y.P.S. Bajaj (Ed.) *Biotechnology in Agriculture and Forestry*, Vol. 30, *Somatic Embryogenesis and Synthetic Seed I*, Springer-Verlag, Berlin Heidelberg, pp. 455-467.

Dumet, D. and Berjak, P. (2002) Cryopreservation of neem (*Azadirachta indica* A. Juss.) seeds. In: L.E. Towill and Y.P.S. Bajaj (Eds.) *Biotechnology in Agriculture and Forestry* ***50***. *Cryopreservation of Plant Germplasm II*. Springer Verlag, Berlin, Heidelberg. pp. 213-219.

Dumet, D., Berjak, P. and Engelmann, F. (1997) Cryopreservation of Zygotic and somatic embryos of tropical species producing recalcitrant or intermediate seeds. In: M.K. Razdan and E.C. Cocking (Eds.), *Conservation of Plant Genetic Resources In Vitro, General aspects*, volume **1**, Science Publishers Inc., USA, Enfield, NH, pp. 153-172.

Dumet, D., Engelmann, F., Chardrillange, N., Duval, Y. and Dereuddre, J. (1993) Importance of sucrose for the acquisition of tolerance to desiccation and cryopreservation of oil palm somatic embryos. *CryoLetters* **14**, pp. 243-250.

Elhag, H.M., Whipkey, A. and Janick, J. (1987) loc. cit. Pence, V.C. (1995) Somatic embryogenesis in Cacao (*Theobroma cacao)*. In: Y.P.S. Bajaj (Ed.) *Biotechnology in Agriculture and Forestry*, Vol. 30, *Somatic Embryogenesis and Synthetic Seed I*, Springer-Verlag, Berlin Heidelberg, pp. 455-467.

Elhag, H.M., Whipkey, A. and Janick, J. (1988) loc. cit. Pence, V.C. (1995) Somatic embryogenesis in Cacao (*Theobroma cacao)*. In: Y.P.S. Bajaj (Ed.) *Biotechnology in Agriculture and Forestry*, Vol. 30, *Somatic Embryogenesis and Synthetic Seed I*, Springer-Verlag, Berlin Heidelberg, pp. 455-467.

Elkonin, A.L. and Pakhomova, N.V. (2000) Influence of nitrogen and phosphorus on induction embryogenic callus of sorghum. *Plant Cell Tissue and Organ Culture* **61**, pp. 115–123.

Ellis, R.H., Hong, T.D. and Roberts, E.H. (1990) An intermediate category of seed storage behaviour? I. Coffee. *Journal of Experimental Botany* **41**, pp. 1167–1174.

Engelmann, F. (1991) *In vitro* conservation of tropical plant germplasm-a review. *Euphytica* **57**, pp. 227-243.

Engelmann, F. (1992) Cryopreservation of embryos. In: Y. Dattée, C. Dumas and A. Gallais (Eds.) *Reproductive Biology and Plant Breeding*, Springer Verlag, Berlin, p. 281-290.

Engelmann, F. (1997) *In vitro* conservation methods. In: J.A. Callow, B.V. Ford-Lloyd and H.J. Newbury (Eds.) *Biotechnology and Plant Genetic Resources*, CAB International, Wallingford, UK, pp. 119-161.

Engelmann, F. (2000) Importance of cryopreservation for the conservation of plant genetic resources. In: Engelmann, F., Takagi, H. (Eds.) *Cryopreservation of tropical plant germplasm. Current research progress and application*. IPGRI, Rome, pp. 8-20.

Essan, E.B. (1977) Micropropagation of cocoa (*Theobroma cacoa* L.). In: Bajaj Y.P.S. (Ed.) *Biotechnology in agriculture and forestry, High-tech and micropropagation II*. volume **18**. Springer, Berlin Heidelberg New York, pp. 96-122.

Essan, E.B. (1981) loc. cit. Pence, V.C. (1995) Somatic embryogenesis in Cacao (*Theobroma cacao*). In: Y.P.S. Bajaj (Ed.) *Biotechnology in Agriculture and Forestry*,

Vol. 30, *Somatic Embryogenesis and Synthetic Seed I*, Springer-Verlag, Berlin Heidelberg, pp. 455-467.

Evans, D.A., Sharp, W.R. and Flick, C.E. (1981) Growth and behaviour of cell cultures. Embryogenesis and organogenesis. In: T.A. Thorpe (Ed.) *Plant Tissue Culture: Methods and Applications in Agriculture.* Academic Press, New York, pp. 45-113.

Fahy, G.M., Macfarlane, D.R., Angell, C.A. and Meryman, H.T. (1984) Vitrification as an approach to cryopreservation. *Cryobiology* **21**, pp. 407-426.

Fang, J., Wetten, A. and Hadley, P. (2003) Cryopreservation of cocoa (*Theobroma cacao* L.) somatic embryos for long-term germplasm storage. *Plant science* **166**, pp. 669-675.

Feher, A., Pasternak, T.P. and Dudits, D. (2003) Transition of somatic plant cells to an embryogenic state. *Plant Cell Tissue and Organ Culture* **74**, pp. 201-228.

Ferreira, E.M., Alfenas, A.C., Maffia, L.A., Mafia, L.G. and Mounteer, A.H. (2008) Effectiveness of systemic fungicides in the control of *Quambalaria eucalypti* and their effects on production of eucalypt mini-cuttings for rooting. *Crop Protection* **27**, pp. 161-170.

Figueira, A. and Janick, J. (1993) Development of nucellar somatic embryos of *Theobroma cacao*. *Acta Horticulturae* **336**, pp. 231-236.

Figueira, A., Whipkey, A. and Janick, J. (1991) Increased CO_2 and light promote *in vitro* shoot growth and development of *Theobroma cacao*. *Journal of the American Society for Horticultural Science* **116**, pp. 585-589.

Flynn, W.P., Glicenstein, L.J. and Fritz, P.J. (1990) *Theobroma cacao* L.: an axillary bud *in vitro* propagation procedure. *Plant Cell Tissue and Organ Culture* **20**, pp. 111-117.

Fu, J.R., Xia, Q.H. and Tang, L.F. (1993) Effects of desiccation on excised embryonic axes of three recalcitrant seeds and studies on cryopreservation. *Seed Science and Technology* **21**, pp. 85-95.

Fu, J.R., Zhang, B.Z., Wang, X.P., Qiao, Y.Z. and Huang, X.L. (1990) Physiological studies on desiccation, wet storage and cryopreservation of recalcitrant seeds of three fruit species and their excised embryonic axes. *Seed Science and Technology* **18**, pp. 743-754.

Gagliardi, R.F., Pacheo, G.P., Carneiro, L.A., Valles, J.F.M., Viera, M.L.C. and Mansur, E. (2003) Cryopreservation of *Arachis* species by vitrification on *in vitro* grown shoot apices and genetic stability of recovered plants, *CryoLetters* **24**, pp. 103-110.

George, E.F. (1993) *Plant Propagation by Tissue Culture. Part 1, The Technology*. Edington: Exegetics Ltd.

George, E.F. (1996) *Plant Propagation by Tissue Culture. Part 2, In Practice*. Edington: Exegetics Ltd.

González-Benito, M.E., Nunez-Moreno, Y. and Martin, C. (1998) A protocol to cryopreserve nodal explants of *Antirrhinum microphyllum* by encapsulation-dehydration. *CryoLetters* **19**, pp. 225-230.

González-Benito, M.E., Prieto, R-M., Herradon, E. and Martin, C. (2002) Cryopreservation of *Quercus suber* and *Quercus ilex* embryonic axes: *In vitro* culture, desiccation and cooling factors. *CryoLetters* **23**, pp. 283-90.

Goveia, M., Kioko, J.I. and Berjak, P. (2004) Developmental status is a critical factor in the selection of excised recalcitrant axes as explants for cryopreservation: a study on *Trichilia dregeana* Sond. *Seed Science Research* **14**, pp. 241-248.

Grout, B.W. (1990) *In vitro* conservation of germplasm. In: S.S. Bhojwani (Ed.) *Plant Tissue Culture: Applications and Limitations*, Elsevier Science Publishers B.V., Netherlands, pp. 394-411.

Guiltinan, M.J., Li, Z., Traore, A. and Maximova, S. (2000) Method and tissue culture media for inducing somatic embryogenesis, Agrobacterium-mediated transformation and efficient regeneration of cacao plants. The Penn State Research Foundation. Online: http://www.freepatentsonline.com/6150587.html.

Haberlandt, G. (1902) Culturversuche mit isolierten Pflanzenzellen. Reprint (2003) In: Laimer, M. and Rucker, W. (Eds.) *Plant tissue culture, 100 years since Gottlieb Haberlandt*, Springer-Verlag, New York, pp. 1-25.

Halmagyi, A., Deliu, C. and Coste, A. (2005) Plant regrowth from potato shoot tips cryopreserved by a combined vitrification-droplet method. *CryoLetters* **26** (5), pp. 313-322.

Harry, I.S. and Thorpe, T.A. (1991) Somatic embryogenesis and plant regeneration from mature zygotic embryos of red spruce. *Botanical Gazette* **152**, pp. 446-452.

Hartmann, H.T., Kester, D.E., Davies, F. and Geneve, R. (2001) Hartmann and Kester's Plant Propagation: Principles and Practices, 7th Edition.

He, S., Liu, C. and Saxena, P.K. (2007) Plant regeneration of an endangered medicinal plant *Hydrastis Canadensis* L. *Scientia horticulturae* **113**, pp. 82-86.

Hicks, G.S. (1980) Patterns of organ development in plant tissue culture and the problem of organ determination. *Botanical review* **46**, pp. 1-23.

Ho, W. and Vasil, I.K. (1983) Somatic embryogenesis in sugarcane (*saccharum officinarum* L.) I. The morphology and physiology of callus formation and the ontogeny of somatic embryos. *Protoplasma* **118**, pp. 169-180.

Holb, I.J. and Schnabel, G. (2007) Differential effect of triazoles on mycelial growth and disease measurements of *Monilinia fructicola* isolates with reduced sensitivity to DMI fungicides. *Crop Protection* **26**, pp. 753-759.

Homdork, S., Fehrmann, H. and Beck, R. (2000) Effects of field application of tebuconazole on yield, yield components and the mycotoxin content of *Fusarium* infected wheat grain. *Journal of Phytopathology* **148**, pp. 1-6.

Hong, T.S. and Ellis, R.H**.** (1998) Contrasting seed storage behaviour among different species of *Meliaceae*. *Seed Science and Technology* **26,** pp. 77-95.

Hussey, G. (1983) *In vitro* propagation of horticultural and agricultural plants. In: S.H. Mantell and H. Smith (Eds.) *Plant biotechnology*. Cambridge, University Press, pp. 111-138.

International Board for Plant Genetic Resources (1984) The potential for using *in vitro* techniques for germplasm collection. IBPGR, Rome.

Iraqi, I. and Tremblay, F.M. (2001) The role of sucrose during maturation of black spruce (*Picea mariana*) and white spruce (*Picea glauca*) somatic embryos. *Physiologia Plantarum* **111** (3), pp. 381-388.

Janick, J. and Whipkey, A. (1985) loc. cit. Traore, A., Maximova, S.N. and Guiltinan, M.J. (2003) Micropropagation of *Theobroma cacao* L. using somatic embryo-derived plants. *In Vitro Celullar and Developmental Biology* **39**, pp. 332-337.

Jiménez, V. M., Castillo, J., Tavares, E., Guevara, E. and Montiel, M. (2006) *In vitro* propagation of the neotropical giant bamboo, *Guadua angustifolia* Kunth, through axillary shoot proliferation. *Plant Cell Tissue and Organ Culture* **86**, pp. 389-395.

Junaid, A., Mujib, A., Bhat, M.A. and Sharma, M.P. (2006) Somatic embryo proliferation, maturation and germination in *Catharanthus roseus*. *Plant Cell Tissue and Organ Culture* **84**, pp. 325-332.

Jun-Yan, Z., Fu-Xing, G. and Razdan, M.K. (2000) Somatic embryogenesis and germplasm conservation of plants. In: M.K. Razdan and E.C. Cocking (Eds.) *Conservation of Plant Genetic Resources In Vitro, Applications and Limitations*, volume 2, Science Publishers, Inc., Enfield, NH, USA, pp. 167-191.

Kamata, T. and Uemura, M. (2004) Solute accumulation in wheat seedlings during cold acclimation: contribution to increased freezing tolerance. *CryoLetters* **25**, pp. 311-322.

Kartha, K.K. (1985) *Cryopreservation of Plant Cells and Organs*, CRC Press, Inc., Boca Raton, Florida.

Kartha, K.K. (1987) Advances in the cryopreservation technology of plant cells and organs. In: C.E., Green, D.A., Somers, W.P., Hackett and D.D., Biesboer (Eds.) *Plant Tissue and Cell Culture.* Alan R. Liss, Inc., New York, pp. 447-458.

Kartha, K.K. and Engelmann, F. (1994) Cryopreservation and Germplasm Storage. In: I.K., Vasil and T.A., Thorpe (Eds.) *Plant Cell and Tissue Culture.* Kluwer Academic Publishers, Dordrecht, pp. 195-230.

Kartsonas, E. and Papafotiou, M. (2007) Mother plant age and seasonal influence on *in vitro* propagation of *Quercus euboica* Pap., an endemic, rare and endangered oak species of Greece. *Plant Cell Tissue and Organ Culture* **90**, pp. 111-116.

Kaur, R., Sharma, N., Kumar, K., Sharma, D.R. and Sharma, S.D. (2006) *In vitro* germination of walnut (*Juglans regia* L.) embryos. *Scientia Horticulturae* **109**(4), pp. 385-388.

Keller, E.R.J. (2005) Improvement of cryopreservation results in garlic using low temperature preculture and high-quality in vitro plantlets. *CryoLetters* **26** (6), pp. 357-366.

Khan, S., Jabbar, A., Hasan, C.M. and Rashid, M.A. (2001) Antibacterial activity of *Barringtonia racemosa*. *Fitoterapia* **72**, pp. 162-164.

Kim, H., Cho, E., Baek, H., Kim, C., Keller, E.R.J. and Engelmann, F. (2004) Cryopreservation of garlic shoot tips by vitrification: Effects of dehydration, rewarming, unloading and regrowth conditions. *Cryoletters* **25**, pp. 59-70.

Kim, Y.W. and Moon, H.K. (2007) Enhancement of somatic embryogenesis and plant regeneration in Japanese larch (*Larix leptolepis*). *Plant Cell Tissue and Organ Culture* **88**, pp. 241-245.

Kintzios, S., Nikalou, A. and Skoula, M. (1999) Somatic embryogenesis and *in vitro* rosmaniric acid accumulation in *Salvia officinalis* and *S. fruticosa* leaf callus cultures. *Plant Cell Reports* **18**, pp. 1-5.

Kioko, J.I., Berjak, P. and Pammenter, N.W. (2003) Responses to dehydration and conservation of the non-orthodox seeds of *Warburgia salutaris*. *South African Journal of Botany* **69**, pp. 532-539.

Kioko, J., Berjak, P., Pammenter, N.W., Watt, M.P. and Wesley-Smith, J. (1998) Desiccation and cryopreservation of embryonic axes of *Trichilia dregeana* Sond. *CryoLetters* **19**, pp. 5-14.

Kioko, J.I., Naidoo, S., Fintel, Von G.T., Berjak, P. and Pammenter N.W. (2003) Post-shedding behaviour and cryopreservation of seeds and embryonic axes of cocoa (*Theobroma cacao* I.) of West African provenance. *Proceedings from the South African Association of Botanists conference*, Durban, South Africa.

Klimaszewska, K. and Cyr, D.R. (2002) Conifer somatic embryogenesis: I. Development. *Dendrobiology* **48**, pp. 31-39.

Kononowicz, H. and Janick J. (1984) loc. cit. Pence, V.C. (1995) Somatic embryogenesis in Cacao (*Theobroma cacao).* In: Y.P.S. Bajaj (Ed.) *Biotechnology in Agriculture and Forestry*, Vol. 30, *Somatic Embryogenesis and Synthetic Seed I*, Springer-Verlag, Berlin Heidelberg, pp. 455-467.

Kononowicz, H., Kononowicz, A.K., and Janick, J. (1984) loc. cit. Pence, V.C. (1995) Somatic embryogenesis in Cacao (*Theobroma cacao).* In: Y.P.S. Bajaj (Ed.) *Biotechnology in Agriculture and Forestry*, Vol. 30, *Somatic Embryogenesis and Synthetic Seed I*, Springer-Verlag, Berlin Heidelberg, pp. 455-467.

Krishnapillay, B. (2000) Towards the use of cryopreservation as a technique for conservation of tropical recalcitrant seeded species. In: M.K. Razdan and E.C. Cocking (Eds.), *Conservation of Plant Genetic Resources In Vitro*, Applications and Limitations. Volume **2**, Science Publishers Inc., USA, Enfield, NH, pp. 139-166.

Lardet, L., Aguilar, M.E., Michaux-Ferriere, N. and Berthouly, M. (1998) Effect of strictly plant-related factors on the response of *Hevea brasiliensis* and *Theobroma cacao* nodal explants cultured *in vitro*. *In Vitro Cellular and Developmental Biology* **34**, pp. 34-40.

Leakey, R.B., Mesén, J.F., Tchoundjeu, Z., Longman, K.A., McPherson Dick, J., Newton, A., Matin, A., Grace, J., Munro, R.C. and Muthoka, P.N. (1990) Low-technology techniques for the vegetative propagation of tropical trees. *Commonwealth Forestry Review* **69**(3), pp. 247-257.

Leunufna, S. and Keller, E.R.J. (2005) Cryopreservation of yams using vitrification modified by including droplet method: Effects of cold acclimation and sucrose. *CryoLetters* **26 (2)**, pp. 93-102.

Levin, R. and Vasil, I.K. (1989) An integrated and automated tissue culture system for mass propagation of plants. *In Vitro Cellular and Developmental Biology* **25**, pp. 21-27.

Li, Z., Traore, A., Maximova, S. and Guiltinan, M. J. (1998) Somatic embryogenesis and plant regeneration from floral explants of cacao (*Theobroma cacao* L.) using thidiazuron. *In Vitro Cellular and Developmental Biology* **34**, pp. 293-299.

Linsmaier, E.M. and Skoog, F. (1965) Organic growth factor requirements of tobacco tissue cultures. *Physiologia Plantarum* **18**, pp. 100-127.

Litz, R.E. (1986) Tissue culture studies with *Theobroma cacoa*. In: P.S. Dimick (Ed.) *Proceedings of the Cacao Biotechnology Symposium*, Pennsylvania State University, University Park, pp. 111-120.

Liu, W., Moore, P.J. and Collins, G.B. (1992) Somatic embryogenesis in soybean via somatic embryo cycling. *In Vitro Cellular and Developmental Biology* **28**, pp. 153-160.

Lloyd, D.G. and McCown, B.H. (1981) Commercially feasible micropropagation of mountain Laural (*Kalmia latifolia)* by use of shoot-tip culture. *Proceedings of the International Plant Propagation Society* **30**, pp. 421-429.

Lopez-Baez, O., Bollon, H., Eskes, A. and Petiard, V. (1993) loc. cit. Pence, V.C. (1995) Somatic embryogenesis in Cacao (*Theobroma cacao)*. In: Y.P.S. Bajaj (Ed.) *Biotechnology in Agriculture and Forestry*, Vol. 30, *Somatic Embryogenesis and Synthetic Seed I*, Springer-Verlag, Berlin Heidelberg, pp. 455-467.

Malaurie, B., Trouslot, M., Engelmann, F. and Chabrillange, N. (1998) Effect of pretreatment conditions on the crypreservation of *in vitro*-cultured yam (*Dioscorea Alata* 'Brazo Fuerte' *and D. bulbifera* 'Noumea Imboro') shoot apices by encapsulation-dehydration. *CryoLetters* **19**, pp.15-26.

Mathews, D. (2004) Studies on *in vitro* regeneration and mutagenesis in Brahmi (*Bacopa monnieri* (L.) PENNELL.) PhD Thesis, Division of Horticulture, University of Agricultural Sciences, Bangalore.

Matsumoto, T. and Sakai, A. (1995) An approach to enhance dehydration tolerance of alginate-coated dried meristems cooled to –196°C. *CryoLetters* **16**, pp. 299–306.

Matu, E.N., Lindsey, K.L. and Van Staden, J. (2006) Micropropagation of *Maytenus senegalensis* (Lam.) Excell. *South African Journal of Botany* **72**, pp. 409-415.

Maunder, M. (2001) Plant conservation. Encyclopedia of Biodiversity, Volume **4**, Academic Press, pp. 645-658.

Maximova, S.N., Alemanno, L., Young, A., Ferriere, N., Traore, A., Guiltinan, M. (2002) Efficiency, genotypic variability, and cellular origin of primary and secondary somatic embryogenesis of *Theobroma cacao* L. *In Vitro Cellular and Developmental Biology - Plant* **38**, pp. 252–259.

Maxted, N., Ford-Lloyd, B.V. and Hawkes, J.G. (1997) Complementary conservation strategies. In: N. Maxted, B.V. Ford-Lloyd and J.G. Hawkes (Eds.) *Plant Genetic Resources Conservation*. Chapman & Hall, London, pp.15-39.

Mayaba, N., Minibayeva, F. and Beckett, R.P. (2002) An oxidative burst of hydrogen peroxide during rehydration following desiccation in the moss *Atrichum androgynum*. *New Phytologist* **155**, pp. 275-283.

Mazur, P. (1984) Freezing of living cells: mechanisms and applications. *American Journal of Physiology* **247**, pp. 125-142.

McAlister, B., Finnie, J., Watt, M.P. and Blakeway, F. (2005) Use of temporary immersion bioreactor system (RITA) for the production of commercial *Eucalyptus* clones at Mondi Forests (SA), *Plant Cell Tissue and Organ Culture* **81**, pp. 1-12.

McKellar, D.S. (1993) Micropropagation and *in vitro* studies of *Pinus patula* Scheide *et* Deppe. Masters thesis, University of KwaZulu-Natal, Durban.

Merkle, S.A., Parrott, W.A. and Williams, E.G. (1990) Applications of somatic embryogenesis and embryo cloning. In: S.S. Bhojwani (Ed) *Plant tissue culture: Applications and Limitations*. Elsevier Science Publishers B.V., Netherlands, pp. 67-101.

Mishra, Y. Patel, P.K., Yadav, S., Shirin, F. and Ansari, S.A. (2007) A micropropagation system for cloning of *Bambusa tulda* Roxb. *Scientia horticulturae*, doi:10.1016/j.scienta.2007.10.002.

Mix-Wagner, G., Conner, A.J. and Cross, R.J. (2000) Survival and recovery of asparagus shoot tips after cryopreservation using the "droplet" method. New Zealand *Journal of Crop and Horticultural Science abstracts* **28**, pp. 289-292.

Mix-Wagner, G. and Schumacher, H.M. (2003) The *in vitro* conservation of valuable genetic resources. In: Laimer, M. and Rucker, W. (Eds.) *Plant tissue culture, 100 years since Gottlieb Haberlandt,* Springer-Verlag, New York, pp. 135-151.

Moges, A.D., Shibli, R.A. and Karam, N.S. (2004) Cryopreservation of African violet (*Saintpaulia ionantha* wendl.) shoot tips. *In Vitro Cellular and Developmental Biology – Plant* **40**, pp. 389-395.

Mohamed, S.V., Sung, J-M., Jeng, T-L. and Wang, C-S. (2006) Organogenesis of *Phaseolus angularis* L.: high efficiency of adventitious shoot regeneration from etiolated seedlings in the presence of N6 benzylaminopurine and thidiazuron. *Plant Cell Tissue and Organ Culture* **86**, pp. 187-199.

Montgomery, M. and Paulsrud, B. (2006) Beyond Strobys and Triazoles: How Do FungicidesWork?Online:http://www.ipmcenters.org/IPMSymposiumV/posters/131.pdf.

Murashige, T. and Skoog, E. (1962) A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiologia Plantarum* **15**, pp. 473-497.

Mycock, D.J. (1999) Addition of calcium and magnesium to a glycerol and sucrose cryoprotectant solution improves the quality of plant embryo recovery from cryostorage. *CryoLetters* **20**, pp. 77-82.

Mycock, D.J. and Berjak, P. (1990) Fungal contaminants associated with several homoiohydrous (recalcitrant) seed species. *Phytophylactica* **22**, pp. 413-418.

Mycock, D.J., Watt, M.P. and Berjak, P. (1991) A simple procedure for the cryopreservation of hydrated embryonic axes of *Pisum sativum*. *Journal of Plant Physiology* **138**, pp. 728-733.

Mycock, D., Wesley-Smith, J. and Berjak, P. (1995) Cryopreservation of somatic embryos of four species with and without cryoprotectants treatment. *Annals of Botany* **75**, pp. 31-336.

Naidoo, P. (2004) Development of storage protocols for *Theobroma cacao*. Honours thesis, University of KwaZulu-Natal, Durban.

Nairn, B.J., Furneaux, R.H. and Stevenson, T.T. (1995) Identification of an agar constituent responsible for hydric control in micropropagation of radiata pine. *Plant Cell Tissue and Organ Culture* **43**, pp. 1–11.

Namasivayam, P. (2007) Acquisition of embryogenic competence during somatic embryogenesis. *Plant Cell Tissue and Organ Culture* **90**, pp. 1-8.

Narayanaswamy, S. (1977) Regeneration of Plants from Tissue Cultures. In: J. Reinert and Y.P.S. Bajaj (Eds.) *Applied and Fundamental Aspects of Plant Cell, Tissue, and Organ Culture*. Springer-Verlag, Berlin Heidelberg New York, pp. 179-248.

Ng, S.Y.C. and Ng, N.Q. (1991) Reduced-growth storage of germplasm. In: J.H Dodds (Ed.), *In vitro Methods for Conservation of Plant Genetic Resources*. Chapman and Hall, London, UK, pp.11-39.

Ngamau, K. (2001) Promoting side shoot development in *Zantedeschia aethiopica* 'Green Goddess'. *Gartenbauwiessenschaft* **66**, pp. 85-92.

Nhut, D.T., Le, B.V. and Van, K.T.T. (2000) Somatic embryogenesis and direct shoot regeneration of rice (*Oryza sativa* L.) using thin cell layer culture of apical meristematic tissue. *Journal of Plant Physiology* **157**(5), pp. 559–565.

Nhut, D.T., Thuy Minh Hanh, N., Quoc Tuan, P., Thi Minh Nguyet, L., Thi Huyen Tram, N., Chung Chinh, N., Nguyen, N.H. and Nang Vinh, D. (2006) Liquid culture as a positive condition to induce and enhance quality and quantity of somatic embryogenesis of *Lilium longiflorum*. *Scientia Horticulturae* **110**(1), pp. 93-97.

Niino,T., Hettiarachchi, A., Takahashi J. and Samarajeewa, P.K. (2000) Cryopreservation of lateral buds of *in vitro* grown innala plants (*Solenostemon rotundifolius*) by vitrification. *Cryoletters* **21,** pp. 349-356.

Novak, F.J., Donini, B. and Owusu, G. (1986) Somatic embryogenesis and *in vitro* plant development of cacoa (*Theobroma cacoa*). In: Proceeding of the International Symposium on Nuclear techniques and *in vitro* culture for plant improvement. IAEA, Vienna, pp. 443-449.

Orchard, J.E., Collin, H.A. and Hardwick, K. (1979) Culture of shoot apices of *Theobroma cacao*. *Physiologia Plantarum* **47**, pp. 207-210.

Pammenter, N.W. and Berjak, P. (1999) A review of recalcitrant seed physiology in relation to desiccation-tolerance mechanisms. *Seed Science Research* **9**, pp. 13-37.

Pammenter, N.W., Berjak, P., Wesley-Smith, J. and Willigen, C.V. (2002) Experimental aspects of drying and recovery. In: M. Black and H.W. Pritchard (Eds.) *Desiccation and Survival in Plants: Drying Without Dying*, CAB International, pp. 93-110.

Pammenter, N.W., Greggains, V., Kioko, J.I., Wesley-Smith, J., Berjak, P. and Finch-Savage, W.E. (1998) Effects of differential drying rates on viability retention of recalcitrant seeds of *Ekebergia capensis. Seed Science Research* **8**, pp. 463-471.

Pammenter, N.W., Vertucci, C.W. and Berjak, P. (1991) Homeohydrous (recalcitrant) seeds: dehydration, the state of water and viability characteristics in *Landolphia kirkii. Plant Physiology* **96**, pp. 1093-1098.

Panis, B., Strosse, H., Van Den Hende, S. and Swennen, R. (2002) Sucrose preculture to simplify cryopreservation of banana meristem cultures. *CryoLetters* **23**, pp. 375-384.

Paranjothy, K., Saxena, S., Banerjee, M., Jaiswal, V.S. and Bhojwani, S.S. (1990) Clonal multiplication of woody perennials. In: S.S. Bhojwani (Ed.) *Plant Tissue Culture: Applications and Limitations*, Elsevier Science Publishers B.V., Netherlands, pp. 161-189.

Park, Y.G. and Son, S.H. (1995) Somatic embryogenesis in Poplars (*Populus nigra* L. x *P. maximowiczii* Henry). In: Y.P.S. Bajaj (Ed.) *Somatic Embryogenesis and Synthetic Seed I, Biotechnology in Agriculture and Forestry* **30**, Springer-Verlag Berlin Heidelberg, pp. 446-454.

Park, Y.S., Lelu-Walter, M.A., Harvengt, L., Trontin, J.F., MacEacheron, I., Klimaszewska, K. and Bonga, J.M. (2006) Initiation of somatic embryogenesis in *Pinus banksiana*, *P. strobes*, *P. pinaster*, and *P. sylvestris* at three laboratories in Canada and France. *Plant Cell Tissue and Organ Cult*ure **86**, pp. 87-101.

Pasqualetto, P.L., Zimmerman, R.H. and Fordham, I. (1988) The influence of cation and gelling agent concentrations on vitrification of apple cultivars *in vitro*. *Plant Cell Tissue and Organ Culture* **14**, pp. 31-40.

Passey, A.J. and Jones, O.P. (1983) Shoot proliferation and rooting *in vitro* of *Theobroma cacao* L. type amelondado. *Journal of Horticultural Science* **58**, pp. 589-592.

Pauku, R.L. (2006) *Barringtonia procera* (cutnut). Species Profiles for Pacific Island Agroforestry. Online: www.traditionaltree.org.

Pence, V.C. (1989) Cacao (*Theobroma cacao* L.). In: Y.P.S Bajaj (Ed) *Biotechnology in Agriculture and Forestry*, Trees II, Volume **5**, Springer-Verlag Berlin Heidelberg, pp. 203-221.

Pence, V.C. (1991a) Cryopreservation of immature embryos of *Theobroma cacao*. *Plant Cell Reports* **10**, pp. 144-147.

Pence, V.C. (1991b) Abscisic acid in developing zygotic embryos of *Theobroma cacao*. *Plant Physiology* **95**, pp. 1291-1293.

Pence, V.C. (1992) Abscisic acid and the maturation of cacao embryos *in vitro*. *Plant Physiology* **98**, pp. 1391-1395.

Pence, V.C. (1995a) Somatic embryogenesis in Cacao (*Theobroma cacao)*. In: Y.P.S. Bajaj (Ed.) *Biotechnology in Agriculture and Forestry*, Vol. 30, *Somatic Embryogenesis and Synthetic Seed I*, Springer-Verlag, Berlin Heidelberg, pp. 455-467.

Pence, V.C. (1995b) Cryopreservation of recalcitrant seeds. In: Y.P.S. Bajaj (Ed) Cryopreservation of Plant Germplasm I-*Biotechnology in Agriculture and Forestry*, Vol. 32. Springer-Verlag Berlin Heidelberg, pp. 43-47.

Pence, V.C., Hasegawa, P.M. and Janick, J. (1979) Asexual embryogenesis in *Theobroma cacao* L. *Journal of the American Society for Horticultural Science* **104**, pp. 145-148.

Pence, V.C., Hasegawa, P.M. and Janick, J. (1980) loc. cit. Pence, V.C (1995) Somatic embryogenesis in Cacao (*Theobroma cacao)*. In: Y.P.S. Bajaj (Ed.) *Biotechnology in Agriculture and Forestry*, Vol. 30, *Somatic Embryogenesis and Synthetic Seed I*, Springer-Verlag, Berlin Heidelberg, pp. 455-467.

Pennycooke, J.C. and Towill, L.E. (2001) Medium alterations improve regrowth of sweet potato (*Ipomoea batatas* {L.} Lam.) Shoot tips cryopreserved by vitrification and encapsulation-dehydration. *CryoLetters* **22**, pp. 381-389.

Perán, R., Berjak, P., Pammenter, N.W. and Kioko, J.I. (2006) Cryopreservation, encapsulation and promotion of shoot production of embryonic axes of a recalcitrant species *Ekebergia capensis* Sparrm. *CryoLetters* **27**, pp. 5-16.

Phartyal, S.S., Thapliyal, R.C., Koedam, N. and Godefroid, S. (2002) *Ex situ* conservation of rare and valuable forest tree species through seed-gene bank. *Current Science* **83**, pp. 1351-1357.

Pond, S.E., Von Aderkas, P. and Bonga, J.M. (2002) Improving tolerance of somatic embryos of *Picea glauca* to flash desiccation with a cold treatment (desiccation after cold acclimation). *In Vitro Cellular and Developmental Biology-Plant* **38**, pp. 341-344.

Potts, S.E. and Lumpkin, T.A. (1997) Cryopreservation of *Wasabia* spp seeds. *CryoLetters* **18**, pp. 185-190.

Preece, J.E., McGranahan, G.H., Long, L.M. and Leslie, C.A. (1995) Somatic embryogenesis in walnut (*Juglans regia*). In: S. Jain, P. Gupta and R. Newton (Eds.) *Somatic Embryogenesis in Woody Plant II*, Kluwer Academic Publishers, Dordrecht, The Netherlands, pp. 99-116.

Puigderrajols, P., Mir, G. and Molinas, M. (2001) Ultrastructure of early secondary embryogenesis by multicellular and unicellular pathways in cork oak (*Quercus suber* L.). *American Journal of Botany* **87**, pp. 179-189.

Quain, M.D., Berjak, P., Acheampong, E. and Kioko, J. (2007) Difficulties in developing successful cryopreservation protocols for shoot tips and nodal bud explants of tropical species. *South African Journal of Botany* **73** (3), pp. 492-493.

Quiroz-Figueroa, F.R., Fuentes-Cerda, C.F.J., Rojas-Herrera, R. and Loyola-Vargas, V.M. (2002) Histological studies on the developmental stages and differentiation of two different somatic embryogenesis systems of *Coffea arabica*. *Plant Cell Reports* **20** (12), pp.1141–1149.

Ramgareeb, S. (1997) Development of protocols for the micropropagation of grasses suitable for the revegetation of acidic mine wastes. Masters thesis, University of KwaZulu Natal, Durban.

Ramon, M., Geuns, J., Swennen, R. and Panis, B. (2002) Polyamines and fatty acids in sucrose precultured banana meristems and correlation with survival rate after cryopreservation. *CryoLetters* **23**, pp. 345–352.

Razdan, M.K. (1993) An Introduction to Plant Tissue Culture. Intercept, Hampchire, UK.

Razdan, M.K. (2003) *Introduction to Plant Tissue Culture*, Second Edition. Science Publishers, Inc., Enfield, USA, pp. 1-365.

Razdan, M.K. and Cocking, E.C. (1997) Biotechnology in conservation of genetic resources. In: M.K. Razdan and E.C. Cocking (Eds.) *Conservation of Plant Genetic Resources In Vitro, General Aspects.*, volume **1**, Science Publishers, Inc., Enfield, NH, USA, pp. 3-25.

Reddy, V., Berjak, P. and Pammenter, N.W. (2007) Assessment of a strategy to curtail fungal proliferation during storage of non-orthodox seeds. *Proceeding from the International Workshop on seed desiccation sensitivity and-tolerance in seeds and vegetative plant tissues*, Drakensburg, South Africa.

Reed, B.M., Schumacher, L., Dumet, D. and Benson, E.E. (2005) Evaluation of a modified encapsulation-dehydration procedure incorporating sucrose pretreatments for the cryopreservation of *Ribes* germplasm. *In Vitro Cellular and Developmental Biology – Plant* **41**, pp. 431-436.

Reinert, J. and Bajaj, Y.P.S (1977) *Applied and Fundamental Aspects of Plant Cell, Tissue, and Organ Culture*. Springer-Verlag, Berlin Heidelberg New York.

Reinhoud, P.J., Van Iren, F. and Kijne, J.W. (2000) Cryopreservation of undifferentiated plant cells. In: F. Engelmann and H. Takagi (Eds.) *Cryopreservation of tropical plant germplasm. Current research progress and application*. IPGRI, Rome, pp. 91-102.

Reuveni, M. and Evenor, D. (2007) On the effect of light on shoot regeneration in petunia. *Plant Cell Tissue and Organ Culture* **89**, pp. 49-54.

Roberts, E.H. (1973) Predicting the storage life of seeds. *Seed Science and Technology* **1**, pp. 499-514.

Saadat, Y.A. and Hennerty, M.J. (2002) Factors affecting the shoot multiplication of Persian walnut (*Juglans regia* L.). *Scientia Horticulturae* **95**, pp. 251-260.

Sahrawat, A.K. and Chand, S. (2001) Continuous somatic embryogenesis and plant regeneration from hypocotyl segments of *Psoralea corylifolia* Linn., an endangered and medicinally important Fabaceae plant. *Current Science* **81** (10), pp. 1328-1331.

Sakai, A. (1995) Cryopreservation of germplasm of woody plants. In: Y.P.S. Bajaj (Ed.) *Biotechnology in Agriculture and Forestry* Vol. 32: Cryopreservation of Plant Germplasm.1, Springer–Verlag, Heidelberg, pp. 53–69.

Sakai, A. (1997) Potentially valuable cryogenic procedures for cryopreservation of cultured plant meristems. In: M.K. Razdan and E.C. Cocking (Eds.) *Conservation of Plant Genetic Resources In Vitro, General Aspects.*, volume **1**, Science Publishers, Inc., Enfield, NH, USA, pp. 53-66.

Sakai, A. (2000) Development of cryopreservation techniques. In: F. Engelmann and H. Takagi (Eds.) *Cryopreservation of tropical plant germplasm. Current research progress and application*. IPGRI, Rome, pp. 1-7.

Sakai, A., Kobayashi, S and Oiyama, I. (1990) Cryopreservation of nuclear cells of navel orange (*Citrus sinensis* osb. Var. *brasiliensis* Tanaka) by vitrification. *Plant Cell Reports* **9**, pp. 30-33.

Salajova, T., Salaj, J. and Kormutak, A. (1999) Initiation of embryogenic tissues and plantlet regeneration from somatic embryos of *Pinus nigra* Arn. *Plant Science* **145**, pp. 33-40.

Salisbury, F.B. and Ross, C.W. (1992) Plant physiology. Wadsworth Publishing Company, Inc. Belmont.

Sanatombi, K. and Sharma, G.J. (2007) Micropropagation of *Capsicum frutescens* L. using axillary shoot explants. *Scientia Horticulturae* **113**, pp. 96-99.

Sant, R., Taylor, M. and Tyagi, A. (2006) Cryopreservation of *in vitro*-grown shoot-tips of Tropical Taro (*Colocasia esculenta* var. *esculenta*) by vitrification. *CryoLetters* **27 (3)**, pp. 133-142.

Santos, R.I. and Stushnoff, C. (2003) Desiccation and freezing tolerance of embryonic axes from *Citrus sinensis* [L.] OSB. pretreated with sucrose. *CryoLetters* **24**, pp. 281-292.

Schemske, D.W., Husband, B.C., Ruckelshaus, M.H., Goodwillie, C., Parker, I.M. and Bishop, J.G. (1994) Evaluating approaches to the conservation of rare and endangered plants. *Ecology* **75**, pp. 584-606.

Schoenweiss, K., Meier-Dinkel, A. and Grotha, R. (2005) Comparison of cryopreservation techniques for long-term storage of Ash (*Fraxinus excelsior* L.). *CryoLetters* **26**, pp. 201-212.

Schofl, U.A. and Zinkernagel, V. (1997) A test method based on microscopic assessments to determine curative and protectant fungicide properties against *Septoria tritici*. *Plant Pathology* **46**, pp. 545–556.

Scowcroft , R. and Larkin, P.J. (1988) Somaclonal variation. In: G. Bock and J. Marsh (Eds.) *Applications of Plant Cell and Tissue Culture*, Ciba Foundation, pp. 21-35.

Seijo, G. (2000) Effects of preculture with sucrose and ABA on cell suspensions water status and its relation with vitrification resistance. *Revista Brasileira de Fisiologia Vegetal* **12**(2), pp. 166-180.

Serala, M., Taulavuori, K., Taulavuori, E., Karhu, J. and Laine K. (2007) Elongation of Scots pine seedlings under blue light depletion is independent of etiolation. *Environmental and Experimental Botany* **60**, pp. 340-343.

Sershen, Pammenter, N.W., Berjak, P. and Wesley-Smith, J. (2007) Cryopreservation of embryonic axes of selected amaryllid species. *CryoLetters* **28**, pp. 387–399.

Sharma, N. and Sharma, B. (2003) Cryopreservation of shoot tips of *Picrorhiza kurroa* Royle ex Benth., an indigenous endangered medicinal plant, through vitrification. *CryoLetters* **24** (10), pp. 181-190.

Shatnawi, M.A., Johnson, K.A. and Torpy, F.R. (2004) *In vitro* propagation and cryostorage of *Syzygium francissi* (Myrtaceae) by the encapsulation–dehydration method. *In Vitro Cellular and Developmental Biology – Plant* **40** (4), pp. 403-407.

Shuler, M.L. (1988) Bioreactors for plant cell culture. In: M. Salomé S. Pais, F. Mavituna and J.M. Novais (Eds.), *Plant Cell Biotechnology*. Springer-Verlag, Berlin Heidelberg, pp. 329-342.

Silvertown, J. (2004) Sustainability in a nutshell. *Trends in Ecology and Evolution* **19** (6), pp. 276-278.

Smith, M.K. and Drew, R.A. (1990) Current applications of tissue culture in plant propagation and improvement. *Australian Journal of Plant Physiology* **17**, pp. 267-89.

Snyman, S.J., Meyer, G.M., Richards, J.R., Ramgareeb, S., Banasiak, M. and Huckett, B. (2007) Use of the temporary immersion RITA® bioreactor system for micropropagation of sugarcane. *South African Journal of Botany* **73**(2), pp. 336-337.

Söndahl, M., Sereduk, T.B., Chen, Z. and Bellato, C.M. (1989) loc. cit. Pence, V.C. (1995) Somatic embryogenesis in Cacao (*Theobroma cacao)*. In: Y.P.S. Bajaj (Ed.) *Biotechnology in Agriculture and Forestry*, Vol. 30, *Somatic Embryogenesis and Synthetic Seed I*, Springer-Verlag, Berlin Heidelberg, pp. 455-467.

Staritsky, G. (1997) Backgrounds and Principles of in *vitro* Conservation of Plant Genetic Resources. In: M.K. Razdan and E.C. Cocking (Eds.), *Conservation of Plant Genetic Resources In Vitro, Vol. 1: General aspects*. Science Publishers Inc., USA, Enfield, NH, pp. 153-172.

Steinmacher, D.A., Clement, C.R. and Guerra, M.P. (2007) Somatic embryogenesis from immature peach palm inflorescence explants: towards development of an efficient protocol. *Plant Cell Tissue and Organ Culture* **89**, pp. 15-22.

Steward, F.C., Mapes, M.O. and Mears, K. (1958) Growth and organized development of cultured cells. Organization in cultures grown from freely suspended cells. *American Journal of Botany* **45**, pp. 705-708.

Stuart, D.A and Strickland, S.G. (1989) Methods and materials for enhanced somatic embryo regeneration in the presence of auxin. United States Plant Genetics, Inc. (Davis, CA) 4818693. Online:http://www.freepatentsonline.com/4818693.html.

Sutherland, J.R., Diekmann, M. and Berjak, P. (2002) *Forest tree seed health for germplasm conservation***.** IPGRI, Rome.

Takagi, H. (2000) Recent developments in cryopreservation of shoot apices of tropical species. In: F. Engelmann and H. Takagi (Eds.) *Cryopreservation of tropical plant germplasm. Current research progress and application*. IPGRI, Rome, pp. 178-193.

Tan, C.L. and Furtek, D.B. (2003) Development of an *in vitro* regeneration system for *Theobroma cacao* from mature tissues. *Plant Science* **164**, pp. 407-412.

Tanaka, D., Niino, T., isuzugawa, K., Hikage, T. and Uemura, M. (2004) Cryopreservation of shoot apices of in vitro grown Gentian plants: Comparison of vitrification and encapsulation-vitrification protocols. *CryoLetters* **25**, pp. 167-176.

Tang, H., Ren, Z. and Krczal, G. (2000) Improvement of English walnut somatic embryo germination and conversion by desiccation treatments and plantlet development by lower medium salts. *In Vitro Cellular and Developmental Biology* **36**, pp. 47-50.

Taylor, M.G. and Vasil, I.K. (1996) The ultrastructure of somatic embryo development in pearl millet (*Pennisetum glaucum*; Poaceae). *American Journal of Botany* **83**, pp. 28-44.

Terzi, M. and Loschiavo, F. (1990) Somatic embryogenesis. In: S.S. Bhojwani (Ed.) *Plant Tissue Culture: Applications and Limitations*, Elsevier Science Publishers B.V., Netherlands, pp. 54-66.

Thakur, R. and Sood, A. (2006) An efficient method for explant sterilization for reduced contamination. *Plant Cell Tissue and Organ Culture* **84**, pp. 369-371.

Thomas, T.J., Panikkar, B., Subramoniam, A., Nair, M.K. and Panikkar, K.R. (2002) Antitumour property and toxicity of *Barringtonia racemosa* Roxb seed extract in mice. *Journal of Ethnopharmacology* **82**, pp. 223-227.

Thorpe, T.A. (1990) The current status of plant tissue culture. In: S.S. Bhojwani (Ed.) *Plant Tissue Culture: Applications and Limitations*, Elsevier Science Publishers B.V., Netherlands, pp. 1-33.

Torres, A.C., Mfe'e, Z.N. and Cantliffe, D.J. (2001) Abscisic acid and osmotic induction of synchronous somatic embryo developments of sweet potato. *In Vitro Cellular and Developmental Biology-Plant* **37**, pp. 262-267.

Towill, L.E. and Forsline, P.L. (1999) Cryopreservation of sour cherry (*Prunus cerasus* L.) using a dormant vegetative bud method. *CryoLetters* **20**, pp. 215–222.

Towill, L.E., Forsline, P.L., Walters, C., Waddell, J.W. and Laufmann, J. (2004) Cryopreservation of *Malus* germplasm using a winter vegetative bud method: results from 1915 accessions. *CryoLetters* **25**, pp. 323-334.

Tran Thanh Van, K. and Trinh, T.H. (1990) Organogenic differentiation. In: S.S. Bhojwani (Ed.) *Plant Tissue Culture: Applications and Limitations*, Elsevier Science Publishers B.V., Netherlands, pp. 33-53.

Traore, A., Maximova, S.N. and Guiltinan, M.J. (2003) Micropropagation of *Theobroma cacao* L. using somatic embryo-derived plants. *In Vitro Cellular and Developmental Biology* **39**, pp. 332-337.

Tsewana, A. (2001) Development of a protocol for the micropropagation of mature *Eucalyptus grandis* clones through somatic embryogenesis. MSc thesis, Technikon Natal, Durban.

Turner, S.R., Touchell, D.H., Senaratna, T., Bunn, E., Tan, B. and Dixon, K.W. (2001) Effects of plant growth regulators on survival and recovery growth following cryopreservation. *CryoLetters* **22**, pp. 163-174.

UNEP (1992) Convention on biological diversity. *Conference Proceedings*, Nairobi, Kenya.

Varghese, D.B., Naidoo, P., Berjak, P. and Pammenter, N.W. (2008) Alternate explants for plant germplasm cryopreservation: A study of selected recalcitrant-seeded species. Proceedings of the 9th ISSS conference on seed Biology, Olsztyn, Poland.

Vasar, V. (2003) Effect of ascorbic acid and citric acid on *ex vitro* rooting and acclimatization of *Prunus avium* L. microshoots. *Acta Horticulturae* **616**, pp. 251-254.

Vertucci, C.W. and Leopold, A.C. (1987) The relationship between water binding and desiccation tolerance in tissues. *Plant Physiology* **85**, pp. 232-238.

Verzeletti, F. (unknown) Cocoa: Cultivation, Breeding and Biotechnology. Online: http://troz.uni-hohenheim.de/teaching/Exkursions/CR04/CRSPS/SPSverzeletti.pdf.

Vidal, N., Sánchez, C., Jorquera, L., Ballester, A. and Vieitez, A.M. (2005) Cryopreservation of chestnut by vitrification of *in vitro*-grown shoot tips. *In Vitro Cellular and Developmental Biology – Plant* **41**, pp. 63-68.

Volk, G.M., Maness, N. and Rotindo, K. (2004) Cryopreservation of garlic (*Allium sativum* L.) using plant vitrification solution 2. *CryoLetters* **25**, pp. 219-226.

Volk, G.M. and Walters C. (2006) Plant vitrification solution 2 lowers water content and alters freezing behavior in shoot tips during cryoprotection*. Cryobiology* **52**, pp. 48-61.

Von Aderkas, P., Thompson, R.G., Zaki, M. and Benkrima, L. (1995) Somatic embryogenesis in Western Larch (*Larix occidentalis*). In: Y.P.S. Bajaj (Ed) *Somatic Embryogenesis and Synthetic Seed I, Biotechnology in Agriculture and Forestry* 30, Springer-Verlag Berlin Heidelburg, pp. 378-387.

Walters, C., Farrant, J.M., Pammenter, N.W. and Berjak, P. (2002) Desiccation Stress and Damage. In: M. Black and H.W. Pritchard (Eds.) *Desiccation and Plant Survival*. CABI Publishing, Wallingford, UK, pp. 263-291.

Walters, C., Pammenter, N.W., Berjak, P. and Crane, J. (2001) Desiccation damage, accelerated ageing and respiration in desiccation tolerant and sensitive seeds. *Seed Science Research* **11**, pp. 135-148.

Walters, C., Wheeler, L. M. and Grotenhuis, J. M. (2005) Longevity of seeds stored in a genebank: species characteristics. *Seed Science Research* **15**, pp. 1-20.

Wang, Q., Laamanen, J., Uosukainen, M. and Valkonen, J.P.T. (2005) Cryopreservation of *in vitro*-grown shoot tips of raspberry (*Rubus idaeus* L) by encapsulation-vitrification and encapsulation-dehydration. *Plant Cell Reports* **24**, pp. 280–288.

Wang, Q., Li, P., Batuman, O., Gafny, R. and Mawassi, M. (2003) Effect of benzyladenine on recovery of cryopreserved shoot tips of grapevine and citrus cultured *in vitro*. *CryoLetters* **24**, pp. 293-302.

Wann, S. (1986) Patterns and protocols for somatic embryogenesis in woody species. *IPC Technical paper series*, The Institute of Paper Chemistry, Appleton, Wisconsin. Online:http://smartech.gatech.edu/bitstream/1853/2749/1/tps-204.pdf.

Watanabe, K. (2000) Effect of post-thaw treatments on viability of cryopreserved plant cells. In: M.K. Razdan and E.C. Cocking (Eds.) *Conservation of Plant Genetic Resources In Vitro, Applications and Limitations*, volume **2**, Science Publishers, Inc., Enfield, NH, USA, pp. 3-19.

Wen, M.C. (1989) loc. cit. Pence, V.C. (1995) Somatic embryogenesis in Cacao (*Theobroma cacao)*. In: Y.P.S. Bajaj (Ed.) *Biotechnology in Agriculture and Forestry*, Vol. 30, *Somatic Embryogenesis and Synthetic Seed I*, Springer-Verlag, Berlin Heidelberg, pp. 455-467.

Wesley-Smith, J. (2001) Freeze-substitution of dehydrated plant tissues: Artefacts of aqueous fixation. *Protoplasma* **218**, pp. 154-167.

Wesley-Smith, J. (2004) Investigations into the responses of axes of recalcitrant seeds to dehydration and cryopreservation, PhD thesis, University of KwaZulu-Natal.

Wesley-Smith J., Vertucci, C.W., Berjak, P., Pammenter, N.W. and Crane, J. (1992) Cryopreservation of desiccation-sensitive axes of *Camellia sinensis* in relation to dehydration, freezing rate and the thermal properties of tissue water. *Journal of Plant Physiology* **140**, 596-604.

Wesley-Smith, J., Walters, C., Berjak, P. and Pammenter, N.W. (1998) A method for the cryopreservation of embryonic axes at ultra-rapid cooling rates. In: M. Marzalina, K.C. Khoo, N. Jayanthi, F.Y. Tsan and B. Krishnapillay (Eds.) *Recalcitrant Seeds*. Proceedings of IUFRO Seed Symposium, Kuala Lumpur, Malaysia, pp. 132-139.

Wesley-Smith, J., Walters, C., Berjak, P. and Pammenter, N.W. (2004) The influence of water content, cooling and warming rate upon survival of embryonic axes of *Poncirus trifoliata* (L.). *CryoLetters* **25**, pp. 129-138.

Wesley-Smith J., Walters C., Pammenter N.W. and Berjak, P. (2001) Interactions of water content, rapid (non-equilibrium) cooling to -196 °C and survival of embryonic axes of *Aesculus hippocastanum* L. seeds. *Cryobiology* **42**, pp.196-206.

Wilhelm, E. (2003) Tissue culture of broad-leafed forest tree species. In: Laimer, M. and Rucker, W. (Eds.) *Plant tissue culture, 100 years since Gottlieb Haberlandt,* Springer-Verlag, New York, pp. 203-216.

Withers, L.A. (1980) Tissue culture storage for genetic conservation. IBPGR Technical Report, *International Board for Plant Genetic Resources*, Rome.

Withers, L.A. (1988) Germplasm preservation. In: A. Wiley (Ed), *Applications of Plant Cell and Tissue culture*. Wiley, Chichester (Ciba Foundation Symposium 137). pp. 163-177.

Withers, L.A. (1989) *In vitro* conservation and germplasm utilization. In: A.H.D. Brown, O.H. Frankel, D.R. Marshall and Williams, J.T. (Eds.) The uses of plant genetic resources, Cambridge University Press, Cambridge, UK., pp. 309-333.

Withers, L.A. and Street, H.E. (1976) The freeze-preservation of plant cell cultures. In: W. Barz, E. Reinhard and M.H. Zenk (Eds.) *Plant Tissue Culture and Its Biotechnological Applications,* Springer Verlag, Berlin, pp. 226-244.

Wu, H.C., du Toit, E.S. and Reinhardt, C.F. (2007) A protocol for direct somatic embryogenesis of *Protea cynaroides* L. using zygotic embryos and cotyledon tissues. *Plant Cell Tissue and Organ Culture* **89**, pp. 217-224.

Yildirim, T., Kaya, Z. and Isik, K. (2006) Induction of embryogenic tissue and maturation of somatic embryos in *Pinus brutia* TEN. *Plant Cell Tissue and Organ Culture* **87**, pp. 67-76.

Zhang, S. and Lemaux, P.G. (2004) Molecular analysis of *in vitro* shoot organogenesis. *Critical Reviews in Plant Sciences* **23**, pp. 325-335.

Zhao, M.A., Xhu, Y.Z., Dhital, S.P., Khu, D.M., Song, Y.S., Wang, M.Y. and Lim, H.T. (2005) An efficient cryopreservation procedure for potato (Solanum tuberosum L.) utilizing the new ice blocking agent. Supercool X1000. *Plant Cell Reports* **24**, pp. 477-481.

Zhao, Y., Wu, Y., Engelmann, F. and Zhou, M. (2001) Cryopreservation of axillary buds of Grape (Vitis vinifera) in vitro plantlets. *CryoLetters* **22**, pp. 321-328.

Zimmerman, J.L. (1993) Somatic embryogenesis: A model for early development in higher plants. *The Plant Cell* **5**, pp. 1411-1423**.**

Ziv, M. (2005) Simple bioreactors for mass propagation of plants. *Plant Cell Tissue and Organ Culture* **81**, pp. 277-285.

Zoglauer, K., Behrendt, U., Rahmat, A., Ross, H. and Taryono (2003) Somatic embryogenesis – the gateway to biotechnology in conifers. In: Laimer, M. and Rucker, W. (Eds.) *Plant tissue culture, 100 years since Gottlieb Haberlandt,* Springer-Verlag, New York, pp. 135-151.