Development of Efficient Experimental Strategies for The Cryopreservation of Problematic Tropical Rain Forest Tree Germplasm

A thesis submitted in partial fulfilment of the requirements of the University of Abertay Dundee for the degree of Doctor of Philosophy

by

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I certify that this thesis is the true and accurate version of the thesis approved by the examiners.

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Declaration

I hereby declare that this thesis has been composed by myself and that it has not been accepted in any previous application for a degree. The work of which it is a record, is my own, unless otherwise stated. All sources of information have been acknowledged by mean of references.

Jayanthi Nadarajan

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Abstract

The rapid loss of the world's tropical rainforest has serious consequences for mankind and global biodiversity. The conservation and sustainable management of tropical rainforests is helped greatly by the long-term storage and predictable availability of germplasm. Cryopreservation is one of the main methods of conservation. However, protocols must be developed quickly since the seeds of many tropical rainforest trees germinate rapidly and are subject to fungal contamination. Their availability is also unpredictable and hence, often, only small samples are available.

This thesis investigates the novel applicability of statistically designed experiments that use less raw materials and can be performed quicker than traditional designs. Taguchi approach was explored for the first time, for the optimisation of cryopreservation protocols. These approaches can save much valuable time and scarce resources without losing important information. This thesis investigates, for the first time, if these techniques are suitable when cryopreserving tropical rainforest germplasm.

A variety of single and mixed-level traditional 'full factorial' experiments are performed to cryopreserve tropical rainforest seed germplasm from orthodox, intermediate and recalcitrant species. Novel statistical analyses including stepwise and binary logistic regression techniques are used in addition to traditional techniques such as analysis of variance and multiple regression are applied to the experimental data. The results of these experiments are compared with carefully chosen subsets (to simulate fractionally replicated experiments) and those from the Taguchi approach.

The cryopreservation experiments in this thesis were applied to four tropical tree species (*Cassia siamea, Koompassia malaccensis, Sterculia cordata* and *Parkia speciosa*). The whole seed, embryos (at different developmental stages) and shoot-tips were used with techniques including a desiccation protocol for orthodox and desiccation and vitrification for intermediate seeds. An encapsulation-vitrification method including novel use of trehalose as a cryoprotectant was developed for a recalcitrant seed. Differential scanning calorimetry was used to elucidate the critical points at which vitrification occurred.

The conclusions of the cryopreservation experiments suggest that performing a fraction of the full factorial experiments saved valuable raw material and time. When the conclusions of the Taguchi and ANOVA approaches differed, the former resulted in a more robust optimal protocol. Hence the Taguchi approach to optimising cryopreservation experiments is recommended.

This approach can be extended to many areas of conservation of flora or fauna when optimal conditions of a large number of factors must be found when time and/or resources are scarce. However, these techniques work best when the experimenter has sufficient prior knowledge to identify those two-way interactions to be investigated.

CHAPTER 1

PROJECT CONCEPT

1.1 General Introduction

The conservation of tropical forests and the genetic resources derived from them is difficult and requires strategic planning with respect to germplasm acquisition, preservation and sustainable management. The majority of tropical tree species have highly complex life cycles, comprising long juvenile phases and irregular seed bearing episodes, with mass flowering and fruiting often occurring only once in 3-7 years (Marzalina and Krishnapillay, 1999; Yap, 1981). Most tropical seed germplasm is only available from remote or distant sampling locations, within a limited time frame (due to the erratic flowering behaviour) and in small-sized sampling lots. Furthermore, soon after falling from their mother tree most tropical tree seeds mature and germinate rapidly making them unsuitable for traditional storage.

Traditional seed storage can be used to store orthodox (Refer Section 2.3) seeds for long periods although attacks by pest and pathogens reduce viability over extended storage periods (Nadarajan, 1999). However, these techniques are not suitable for the conservation of many tropical rain forest tree seeds especially for those with recalcitrant (Refer Section 2.3) behaviour (Berjak *et al.* 1999). Instead, new approaches for the conservation of forestry germplasm including using tissue culture, pollen storage, DNA banks and cryopreservation have proved successful (Callow *et al.*, 1997).

Seed storage is the most effective and efficient method for the *ex situ* conservation of plant genetic resources. However, there is evidence that cryopreservation in liquid nitrogen improves seed storage longevity (Stanwood and Sowa, 1995; Lakhanpaul *et al.*, 1996). Existing evidence clearly shows that degradation is slower and shelf-life enhanced when cells are stored at cryogenic rather than refrigerator temperatures (Walters *et al.*, 2004). Therefore, an integrated conservation approach which considers all the biological and environmental problems in tropical forestry is necessary.

This thesis examined the feasibility of using efficiently designed experiments which are constructed to aid cryopreservation protocol

development of four tropical forest tree species representing orthodox, intermediate and recalcitrant seed categories.

1.2 Project Motivation

Cryopreservation is one of the most important *ex situ* conservation methods for tropical rain forest tree species. The motivation behind this project is that since, typically, tropical rain forest seeds deteriorate rapidly and are available in small sampling lots, the optimal cryopreservation protocol must be developed rapidly whilst sacrificing as few seeds as possible. A standard approach is to conduct a full factorial experiment (Refer to Section A1.4) and to use analysis of variance (ANOVA) to determine significant main effects and interactions (Refer to Section A1.4.4). This approach has two main disadvantages. Firstly, the full factorial experiment requires all combinations of factor levels to be undertaken to allow all interactions to be investigated. Secondly, ANOVA finds effects that significantly influence the mean of a response variable which may not be the most suitable metric as outliers can influence it, leading to non-robust optimal solutions. In this study, the novel application of Taguchi methods (Refer Chapter 4) as an alternative strategy for designing and analysing experiments to optimise cryopreservation protocols forms a major part of investigation. It is particularly useful when germplasm is in short supply (as with endangered species) and/or experiments must be conducted quickly (as in the case of recalcitrant tree seeds) or a combination of both.

Vitrification-based cryopreservation methods have been increasingly used for intermediate and recalcitrant seed categories. Therefore, the cryobiological component of the study places emphasis on the application of novel statistical experimental designs in optimising vitrification-based cryopreservation protocols. Comparisons of different cryoprotective strategies are therefore undertaken. Finally, as seed storage recalcitrance remains one of the major limiting factors in the uptake of cryopreservation for tropical species, the study also explores the application of Differential Scanning Calorimetry in the evaluation of critical factors in recalcitrant seed cryopreservation and water status.

1.3 Thesis Aims

1) To develop cryopreservation protocols for selected tropical tree species from orthodox, intermediate and recalcitrant seed categories using whole seeds, zygotic embryos and shoot-tips using desiccation, vitrification and encapsulation-vitrification cryoprotectant strategies
2) To compare a range of single and mixed level traditional full factorial, fractionally replicated and Taguchi experiments to obtain an efficient experimental design to cryopreserve the above germplasm
3) To incorporate the above investigations to minimise the number of seeds used to yield the greatest and most accurate interpretation of cryo-conservation responses and thereby develop new approaches for conserving tropical rainforest tree species.
4) To apply Differential Scanning Calorimetry fundamental studies to investigate water status in cryopreserved recalcitrant material.
5) To construct recommendations for the future development of cryopreservation protocols for tropical rainforest germplasm.

1.4 Project Overview

The PhD programme was planned where, as the experiments progressed, there were increasing complexities in the germplasm cryopreserved, cryopreservation method, experimental designs and data analysis applied. The first experiment was designed as the most simple two level factorial experiment (Refer Section A1.4) using orthodox seed, which is amenable to cryopreservation (Pammenter and Berjak, 1999). A simple traditional cryopreservation protocol using desiccation as a cryoprotective strategy was applied for this seed category. The second experiment, a confirmatory experiment to experiment one, but using a more complex three cubic experiment (Refer Section A1.6) was conducted. This was followed by cryopreserving intermediate and recalcitrant seed categories, which are more problematic to cryopreserve. Thus, more complicated mixed-level experiments (Refer Section A1.7) were used for intermediate and recalcitrant seeds in subsequent experiments. In the case of intermediate seed cryopreservation, contemporary vitrification methods were applied

incorporating a range of treatments and additives. For this seed category, both seed and excised zygotic embryos were compared for cryopreservation responses for one experiment and the effect of seed developmental stage was tested in another experiment using the traditional desiccation method. Chemical vitrification using Plant Vitrification Solution Two (PVS2) was also applied to the intermediate seed category (Sakai, 2004). For the recalcitrant seed, a combination of encapsulation-vitrification was applied. In addition, shoot tips were explored as an alternative subject for cryopreservation. Different statistical data analyses were applied for different designs of experiments as summarised in Table 1.1. Seed categories, experimental designs, methods of analysis, material cryopreserved and cryopreservation protocols used in all experiments are also summarised.

1.5 Thesis Structure

Chapter one introduces the project concept of this thesis and Chapter two presents an introduction and review of plant cryopreservation focusing on tropical tree species.

Chapter three introduces the factorial experiments at two, three and mixed-levels, and also describes methods to design fractional factorial experiments.

With Taguchi design being introduced to cryopreservation experiments for the first time, a previous literature research on this subject is considered essential to assist the non-statistically specialised readers. Therefore, Chapter four introduces the theoretical background of the Taguchi method and explains how Taguchi experiments are designed using orthogonal arrays and linear graphs. Analysis of Taguchi experiments using signal to noise ratios (SNR) and three available SNRs for different types of response variables are also introduced with examples.

Chapter five introduces the regression techniques used in this thesis with relevant examples on their application to cryopreservation and their interpretation by data analysis.

Chapters six, seven and eight contain the design, analysis and conclusions of the cryopreservation experiments for orthodox, intermediate and recalcitrant seed categories respectively. In these chapters, the comparisons of full factorial, fractional and Taguchi experiments were made. Differences in data analysis using traditional ANOVA and SNRs are also investigated. Applications of different data analysis methods and their interpretation in obtaining conclusions are incorporated in these chapters. These case studies show the practical usability of the implemented experimental design and data analysis in actual cryopreservation experiments.

Chapter nine presents the general discussion of this work and details future work. This includes proposing future experiments and the uses of different types of experimental design and data analysis in cryopreservation and other wider conservation strategies.

Table 1.1 Summary of experiments

Experiment	Species	Seed category	Design of Experiment	Method of analysis	Explant cryopreserved	Cryopreservation Technique	Section in Thesis
1	*Cassia siamea*	Orthodox	2^5, 2^{5-1}, 2^{5-2}, Taguchi L_{32}, L_{16} and L_8	ANOVA Regression analysis	Whole seed	Desiccation	6.4
2	*Cassia siamea*	Orthodox	3^3, 3^{3-1}, Taguchi L_{27}, and L_9	ANCOVA Regression analysis	Whole seed	Desiccation	6.5
3	*Koompassia malaccensis*	Intermediate	4 x 2^3 Taguchi L_{16} and L_8	ANOVA Regression analysis Covariance analysis	Whole seed and zygotic embryo	Desiccation	7.2
4	*Sterculia cordata*	Intermediate	3^3, 3^{3-1}, Taguchi L_{27}, L_9 and 2 x 3^3, Taguchi L_{18}	ANOVA Regression analysis ANCOVA	Zygotic embryo	Desiccation with investigation of different seed developmental stages	7.3
5	*Sterculia cordata*	Intermediate	2 x 3^3	ANCOVA Binary Logistic Regression Analysis	Zygotic embryo	Vitrification using PVS2	7.4
6	*Parkia speciosa*	Recalcitrant	2 x 3^3	Binary Logistic Regression Analysis	Shoot tips	Encapsulation-vitrification	8.0

CHAPTER 2

INTRODUCTION TO TROPICAL RAINFOREST CRYOPRESERVATION

2.1 Tropical Rainforest Conservation

This chapter reviews the conservation of tropical forest tree germplasm and their constraints. It starts from emphasising the importance of cryopreservation as a part of *ex-situ* conservation programmes for tropical forest tree germplasm. This chapter also introduces three categories of seeds and their amenability to cryopreservation, using the different cryopreservation methods presently available. The application of thermal analysis using Differential Scanning Calorimetry as an aid to cryopreservation protocol development is considered.

Tropical rain forests contain bioresources that play a vital role in maintaining global environmental stability. However, the uncontrolled exploitation of tropical forests endangers plant and animal species threatening biodiversity, socio-economies, ecology and the environment (Uyoh *et al.*, 2003). The rate of loss of this diversity is high due to deforestation, ecosystem fragmentation and overexploitation. The Food and Agricultural Organisation (2001) reported that global forest cover was shrinking at a rate of around 9 million hectares per year. There is a general consensus that tropical rain forests should be conserved and managed on a sustainable basis (Kerr and Burkey, 2002).

Conservation strategies are basically divided into two areas: namely *in situ* and *ex situ* (UNCED, 1992; International Plant Genetic Resources Institute, 2001). *In situ* conservation is defined as 'the conservation of ecosystems and natural habitats and the maintenance and recovery of viable populations of species in their natural surroundings (UNCED, 1992). Conserving the areas where populations of species exist naturally is an underlying condition for the conservation of biodiversity. It can occur in different forms, and entire biomes containing animal and plant ecosystems can be designated as locations for preservation. This type of preservation is extremely important in slowing extinction rates for tropical forests, or controlling timber reserves in forests; but

in general, has little impact on the *ex situ* conservation of genetic resources (Engelmann and Engel, 2002).

In situ conservation can be further divided into three areas (UNEP, 1992). Firstly, a protected region which is a geographically defined area that is designated or regulated and managed to achieve specific conservation objectives. This includes national parks and nature reserves, sustainable use reserves, wilderness areas and heritage sites. Secondly, those which allow a range of economic activities to be undertaken by indigenous peoples in extractive reserves and national forests, and thirdly, where local people act as custodians for the traditional progenitors and varieties and selections contained in home gardens and farms (Jarvis *et al.*, 2000). An interesting aspect of *in situ* conservation is that the genetic pool is not 'frozen' but rather is under constant genetic change due to both natural and artificial selection.

Ex situ conservation is 'conservation of components of biological diversity outside their natural habitats' (Engelmann, 2000b; Phartyal *et al.*, 2002). This includes conservation in seed banks, field genebanks and/or botanical gardens and germplasm repositories. *Ex situ* conservation techniques are particularly appropriate for the conservation of crops and their wild relatives, while *in situ* conservation is especially appropriate for wild species and for landrace materials held on farms (Engels and Wood, 1999).

Efficient conservation of plant genetic resources can best be achieved through an appropriate combination of *in situ* and *ex situ* methods (International Plant Genetic Resources Institute, 2001). However, the acquisition and conservation of genetic resources found within tropical forests is difficult and requires strategic planning.

2.2 Seed Biology and Storage

The main method of conserving plant genetic resources *ex situ* is seed storage (Engelmann, 2000b). Knowledge of seed biology is crucial for proper germplasm handling, including seed storage. In this context, the term 'seed' usually refers to the unit extracted from the fruit and handled as a unit during storage, pre-treatment and sowing. Seed handling encompasses a series of procedures beginning with the selection of the best quality of seed source, through to collection, processing, storage and pre-treatment, to germination (Hay and Probert, 2000). Each link of this chain implies a potential risk of losing seed, and any link in the process is of great importance. If a seed dies due to careless handling during collection or processing, even the best storage conditions will not bring it 'back to life'.

2.3 Seed Classification and Characteristics

Roberts (1973) classified seeds as orthodox or recalcitrant based on their physiological responses to moisture content and temperature and survival during storage. Orthodox seeds can be dried to low moisture of 1-5% without damage and their storability increases with a decrease in seed moisture and storage temperature (Hong and Ellis, 1996). For example, *Dendrocalamus membranaceus* seeds showed increased longevity with decreasing moisture content and storage temperature (Rawat and Thapliyal, 2003). Recalcitrant seeds, on the other hand cannot survive desiccation below a comparatively high moisture of 12-30% (Roberts, 1973). Moreover, even at high moisture, survival is relatively short, and for many species chilling damage occurs at temperatures below or about 15°C (Berjak and Pammenter, 1997). Recalcitrant seeds must therefore be stored at moisture levels above the `lowest-safe' or 'critical' moisture content at which considerable decline in seed viability occurs. Critical moisture contents vary greatly among species and even among cultivars and seed lots. For example, they can vary from 9% and 4% for *Dipterocarpus alatus* and *Dipterocarpus intricatus* respectively (Krishnapillay *et al.,* 1992) to 26% for

Hopea odorata (Nadarajan, 1999) although they belong to the same family Dipterocarpaceae.

Roberts and King (1980) suggested that orthodox species originate from environments subjected to occasional or seasonal drought where the ability to tolerate desiccation is essential for seed survival. On the other hand, recalcitrant species usually originate from moist or aquatic ecosystems in which seeds are subjected to constant high humidity during seed development, maturation and after shedding. This hypothesis has been updated to take into account the intermediate category of seed storage behaviour (Ellis *et al.*, 1990).

Definition of intermediate seed storage behaviour is based on the response of longevity to storage environment (Ouedraogo *et al.*, 1996). Like recalcitrant seeds, they are shed from their mother trees at a relatively high moisture content (25-70%) compared to orthodox seeds. Therefore, they contain a higher amount of cellular free water and hence are more sensitive to desiccation and low temperatures (Goveia *et al.*, 2004). In seeds showing intermediate seed storage behaviour, the trend towards increasing longevity the lower the seed storage moisture content is reversed at a comparatively high moisture content, whereby, at lower moisture contents longevity is reduced (Ellis *et al.*, 1990). But in such species, it is also observed that seeds may be damaged immediately by desiccation at relatively low moisture contents (about seven to 12 percent) depending on species. The critical moisture contents of intermediate seeds below which a more rapid loss in viability occurs during hermetic storage varies considerably with species, degree of maturity, method of seed extraction and post harvest handling. Kundu *et al.* (2003) showed that *Phoebe goalparensis* seed exhibits intermediate storage behaviour where it can be stored at 33% moisture content for 12 months at 5°C temperature with 70% viability. Seeds falling into the intermediate category, as defined by Hong and Ellis (1996), are considered to occupy different positions on the continuum between true recalcitrance and orthodoxy (Berjak and Pammenter, 1994).

It can be argued whether it is possible to categorise all species into these three groups or whether the groups form a continuum from very orthodox seed to very recalcitrant seed. From a practical point of view, it has been established that there are two very important factors concerning seed storage: seed moisture content and storage temperature. For tropical seed it is difficult to ascertain difference between intermediate and recalcitrant categories. The seed storage behaviour also can change through developmental stages for one species, where at early stage of development it might show intermediate category but at full maturity it might exhibit recalcitrant behaviour. The excised embryos and the whole seed of one species also might exhibit different (either intermediate or recalcitrant) storage category (Normah and Vengadasalam, 1992; Marzalina, 1995). Table 2.1. gives the current accepted definitions of the seed categories for tropical seeds (Thomsen, 2000).

Table 2.1. Characteristics of orthodox versus recalcitrant/intermediate seed (Thomsen, 2000)

Orthodox seed	**Recalcitrant and intermediate seed**
Low moisture content at shedding	Relatively high moisture content at shedding
Includes dormant and non-dormant species	Usually non-dormant species
Perennial, woody or annual, herbaceous species	Mostly perennial, woody species
Found in all ecosystems	Often found in humid ecosystems
Usually small seeds	Often large, fleshy seeds

In this thesis, seed and embryos from three different categories (orthodox, intermediate and recalcitrant) were selected for cryopreservation protocol development studies.

2.4 Seed Development

Once the ovule has been fertilised, a chain of processes starts, which potentially leads to the formation of a ripe or mature fruit or seed-bearing organ containing mature germinable seeds although germination may be impeded by the development of dormancy (Bradford, 2002). For most species the maturity of fruit and seed coincides with dispersal. However, some seeds contain physiologically immature embryos at dispersal, for example *Ceiba pentandra* (Lima *et al.*, 2005). These seeds are not germinable at fruit maturity but require after-ripening. The phenomenon is often classified as dormancy. On the other hand, seeds may mature well in advance of fruit maturity for example in *Arabidopsis thaliana* seeds (Raghavan, 2002).

The late events in seed maturation include formation of storage proteins and hormones, and dehydration in orthodox seeds (Schmidt, 2000). In dry fruits, dehydration of seeds is concurrent with the general dehydration of the fruits. In fleshy fruits, dehydration is the result of the increased osmotic pressure due to sugar formation in the fruit pulp. The final moisture content in the seeds depends on species and the external environment (Lima and Ellis, 2005). It has important implications for the storage properties of the seeds. Orthodox seeds typically dry to 5-10% during maturation, whilst, recalcitrant and intermediate seeds maintain relatively high moisture content usually above 40-50% during maturation (Greggains *et al.*, 2000; Bradford, 2002).

Although recalcitrance is predominant in some tropical plant families such as the Dipterocarpaceae, there seems to be no phytogenetic pattern and often two closely related species show very different seed storage behaviour (Thomsen, 2000). If a species is never exposed to low temperatures in the area of natural distribution, temperature sensitivity should be expected. *Hopea odorata* is a typical recalcitrant species from the humid tropics. The seed of *H. odorata* is very desiccation sensitive and does not tolerate temperatures below 15°C (Nadarajan, 1999); while *Quercus robur*, a typical temperate recalcitrant species is desiccation sensitive, but tolerates storage temperatures of -2°C

(Thomsen 2000). Thus, a clue to the behaviour of the seed can probably be found in the natural environment of the species.

2.5 Constraints in Tropical Forest Conservation

Tropical rain forest tree germplasm is some of the most difficult to conserve and requires strategic and complex planning with respect to coordinating its acquisition and conservation (Marzalina and Krishnapillay, 1999). Insufficient baseline information about indigenous tree species and the intermittent availability of seeds and seedlings, are major constraints for the deployment of conservation strategies (Dulloo *et al.*, 1998). Access to seeds and seedlings are in general associated with seed handling and storage problems, which limit the use of many potentially high value indigenous species in tree planting and conservation programmes.

Little is known about the seed physiology of many tropical forest tree species. In many tropical timber species, flowering and fruiting seasons are irregular (Yap and Chang, 1990). A preliminary analysis of phenological observations made in Peninsular Malaysia since 1977 indicates that some species tend to flower and fruit annually, some biennially, and others only once every three or four years (Ng, 1977; Yap and Chang, 1990). Records also indicate that the same species may fruit in different part of the country during different years. However, during a mass fruiting season, the tendency is for all of the different species to flower and fruit almost simultaneously throughout the entire country. Mass flowering and fruiting often occur only once every seven or eight years (Marzalina *et al.*, 1994).

Tropical seed germplasm tends to be only available from remote or distant sampling locations, within a limited time frame and in small-sized sampling lots (Marzalina and Krishnapillay, 1999; Uyoh *et al.*, 2003; Phartyal *et al.*, 2002). The availability of seed is still a severe constraint to good reforestation and conservation programmes. To obtain the best quality seeds germplasm needs to be collected from trees and not from the ground since soon

after falling from mother trees most seeds mature and germinate rapidly, making them unsuitable for traditional storage. They are also very prone to fungal infection making the seeds unsuitable for storage and this is made more problematic with the competition for food sources between small mammals (e.g. squirrels), monkeys and birds that feed on their fleshy cotyledons (Theilade and Petri, 2003).

Another major problem for humid tropical forest tree species conservation is that more than 70% of them have seeds with recalcitrant or intermediate seed storage behaviour which are difficult to store (Ouedraogo *et al.*, 1999). Many of these seeds are sensitive to both desiccation and low temperatures and, consequently, do not tolerate being dried to a low moisture content and cannot be stored at low temperatures for long periods of time (Pammenter *et al.*, 1991).

The use and conservation of a particular tree species is often limited by problems related to seed collection and storage. *Azadirachta indica* seeds for instance can have a limited 'shelf life' viability, which makes immediate sowing necessary and often at times not optimal for seedling survival (Sacande, 2000). *A. indica* requires specialised seed handling and this is also key to the increased use and conservation of many species, particularly for those with non-orthodox seed storage behaviour.

To summarise, environmental and life cycle factors present major practical and technical constraints that limit the application of seed conservation strategies based on standard seed banking criteria to tropical forest tree seeds (Benson, 2004).

2.6 Biotechnology and Forest Conservation

Conventional seed storage is used to support sustainable utilisation programmes and applied for short-term (less than one year) for recalcitrant seeds and longer term periods (more than 5 years) in the case of orthodox seeds (Engelmann and Engels, 2002). However, several serious problems are encountered with conventional storage, in particular, attacks by pest and pathogens which reduce viability over extended storage periods (Pammenter and Berjak, 1999). Therefore, these techniques are not suitable for the conservation of many tropical rain forest tree seeds, especially for those with recalcitrant behaviour. Instead, new biotechnological approaches for the *in vitro* conservation of forestry germplasm including using tissue culture, pollen storage and gene or DNA banks have proved successful (Engelmann, 2004; Marzalina and Krishnapillay, 1999; Benson, 2004). These provide a broad range of possibilities for the protection and conservation of forestry resources (Marzalina and Krishnapillay, 1999; Phartyal *et al.*, 2002). Collected plants are normally stored either in active gene banks containing material that is kept ready for distribution, evaluation or exchange; or as base collections containing duplicates that are kept for future use (long term) or 'emergency' material in case of loss from the active gene banks (Uyoh *et al.*, 2003). Base collections can be maintained under ultra low temperature using cryopreservation.

2.7 Cryopreservation of Tropical Germplasm

Cryopreservation is defined as storage in liquid nitrogen (LN) at –196°C at which point cell division and consequently growth and all other biological activities are completely arrested (Sakai, 2004). This is done in a manner that the viability of the stored material is retained and biological functions and growth can be reactivated after rewarming. Cryopreservation is useful for various types of plant material including whole seeds, embryos, suspension cells, callus, protoplast cultures, gametes and meristems (Touchell, 2000; Turner *et al.*, 2000; Engelmann, 2004). Cryopreserved germplasm appear to

remain genetically stable and this storage also prevents the development of chromosomal aberration, which generally occurs during storage by conventional methods (Harding, 2004; Zhai *et al.*, 2003; Hao *et al.*, 2002). Successful cryopreservation depends upon the application of cryoprotective treatments, without which living cells undergo lethal injury.

During the last decade, the Seed Technology Section at the Forest Research Institute of Malaysia (FRIM) has cryopreserved forest tree germplasm using seeds and embryonic axes. Whole seeds of orthodox and intermediate species can be cryopreserved effectively (Krishnapillay, 2000; Nadarajan, 1999; Marzalina and Normah, 2001; Benson *et al.*, 1996a). However, only low viabilities for cryopreserved embryos of tropical rainforest recalcitrant seeds were recorded (Nadarajan, 1999). Development of reliable protocols for the cryopreservation and subsequent plant generation are the basic requirements together with their integration with stringent collecting and seed handling strategies, as applied to tropical forest tree germplasm.

Nevertheless, a wide variety of temperate plant germplasm systems has been cryopreserved (Benson, 2004). However, cryopreservation protocols, with few exceptions, are still in the developmental stage for many tropical species. For both temperate and tropical species, the application of cryopreservation to tree genetic resources requires special consideration, most particularly for tropical species (Engelmann, 2000a; Benson, 1999; Normah *et al.*, 1996).

Cryopreservation techniques can be divided into two categories depending on whether ice is allowed to form during cooling of the samples (Engelmann, 2004).

2.8 Traditional Cryopreservation

The traditional cryopreservation technique which allows ice to form in cells during cooling, involve preculture of samples, cryoprotection, slow cooling

(0.5-2.0°Cmin^{-1}) to a determined prefreezing temperature (usually –40°C), rapid immersion in liquid nitrogen, storage and rapid warming. With temperature reduction during slow cooling, cells and external medium initially supercool, followed by ice formation in the medium (Mazur, 1984). The cell membranes act as a physical barrier and prevent ice from seeding the cell interior and the cells remain unfrozen but supercooled (Engelmann, 2004). As the temperature is further decreased, an increasing amount of the extracellular solution is converted into ice, thus resulting in the concentration of intracellular solutes. Since cells remain supercooled and their aqueous vapour pressure exceeds that of the frozen external compartment, cells equilibrate by loss of water to external ice. Depending upon the rate of cooling and the prefreezing temperature, different amounts of water will leave the cells before the intracellular contents solidify. In optimal conditions, most or all intracellular freezable water is removed, thus reducing or avoiding detrimental intracellular ice formation upon subsequent immersion in liquid nitrogen. However, too intense freeze-induced dehydration can impose different damaging events due to concentration of intracellular salts and changes in the cell membrane (Meryman *et al.*, 1997). Subsequent rewarming needs to be as rapid as possible to avoid recrystallisation in which ice melts and reforms a larger and more damaging crystal size (Mazur, 2004).

Traditional plant cryopreservation also involves the application of one or more chemical cryoprotective additives categorised as either non-penetrating or penetrating (Benson, 2004). The former (e.g. sucrose, poly-alcohols) withdraw water osmotically from the cell, thus reducing the amount of water available for ice formation. Penetrating cryoprotectants, (usually dimethyl sulphoxide (DMSO)) offer colligative cryoprotection by protecting the cell from the damaging solution effects that the migration of water causes and are applied in combination with controlled rate freezing. The incorporation of preculture additives that dehydrate the cells (mannitol, sorbitol) and pre-treatment steps that simulate cold hardening, or the use of additives known to enhance stress tolerance such as proline, are also incorporated into standard protocols for both dedifferentiated cells and organised structures (Benson, 1994; 1995).

One of the most widely applicable 'traditional' plant cell cryopreservation protocols developed was that of Withers and King (1980). This method utilises a simple controlled-rate freezing unit comprising a methanol bath that allowed solvent temperature reduction to be programmed. Cells are first pre-treated with mannitol, which osmotically removes water prior to the cryoprotection *per se*. They are then cryoproptected in a mixture of DMSO, glycerol and sucrose or DMSO, glycerol and proline and then cooled at a rate of –1°C/min to a terminal transfer temperature of –30°C where they are held for 30-40 minutes before plunging into liquid nitrogen at –196°C. This basic protocol has been modified (Benson, 1994; 1995) and optimised for a number of plant cell systems. Today, computer controlled programmable freezers are used to control the cooling rate of plant, cells, tissues and organs cryopreserved using the traditional approach.

Traditional cryopreservation protocols have been successfully applied to undifferentiated culture systems such as cell suspensions and calluses (Withers and Engelmann, 1998). Protocols using controlled rate freezing have also been applied with success to organised plant structures such as meristems (Benson, 1995; Reed and Chang, 1997). In this thesis, traditional cryopreservation was applied to *Cassia siamea*, an orthodox seed producing species (Section 6.4).

2.9 Contemporary Cryopreservation

Contemporary cryopreservation techniques are increasingly based on the vitrification-based procedure (Benson, 1999). In this technique, tissues are dehydrated using evaporative and/or osmotic desiccation, followed by rapid cooling. As a result all factors that affect intracellular ice formation are avoided. A major advantage of the vitrification-based method is that it is a rapid method, which does not require controlled freezing equipment or sophisticated and expensive apparatus. By precluding ice formation in the cell system, vitrification-based procedures are operationally less complex compared with traditional controlled rate freezing methods and have greater potential for broad applicability (Hirai *et al.*, 1998).

2.9.1 Vitrification

The process of vitrification is now becoming one of the main approaches in the cryopreservation of plant germplasm for long-term conservation. Vitrification refers to the physical process by which a concentrated aqueous solution solidifies into stable amorphous glass at a sufficiently low temperature without ice crystallisation (Sakai, 2004). Avoidance of ice by vitrification can be generally achieved with one of two approaches or a combination of both. The first approach employs cooling highly concentrated vitrification solutions that become sufficiently viscous at low temperatures to suppress crystallisation rates (Taylor *et al.*, 2004; Sakai, 2004). Secondly, vitrification can also be achieved by selecting sufficiently high cooling rates to prevent ice crystallisation in relatively dilute solutions (Mazur, 2004). The second approach generally produces a metastable state that is at risk of devitrification (recrystallisation) during warming.

2.9.2 The physics of vitrification

Molecules in a liquid undergo random, Brownian motion. For freezing to occur in a supercooled liquid, the diffusing molecules must spontaneously form a small cluster (called a nucleus or embryo) of molecules that temporarily has a structure similar to that of ice (Muldrew *et al.*, 2004). In a supercooled liquid such clusters form and dissipate rapidly. If however the cluster is larger than some critical size, it becomes energetically favourable for other diffusing molecules to join the structure, and it grows through the sample (crystallising, or freezing). This process is called nucleation and crystal growth (Mazur, 2004). Nucleation can be either homogeneous (as described above) or heterogeneous, where an impurity (or the container wall) forms a substrate upon which nuclei can grow (Mazur, 2004).

The probability of nucleation in a supercooled liquid depends on several factors: it increases with the volume of the sample, the degree of supercooling; it decreases with increasing solution concentration; and it also increases in the presence of impurities that can act as heterogeneous nuclei (Acker and

McGann, 2001). A pure liquid in a small volume with no impurities can be supercooled below its equilibrium freezing point. Small volumes (microlitres) of pure water, for example, can be cooled to about -40°C the temperature of homogenous nucleation (Mishima and Stanley, 1998). At very low temperatures, viscosity of a solution rises sharply, and molecular diffusion is reduced. If cooling is fast, then the viscosity rises rapidly, hindering nucleation (Muldrew *et al.*, 2004). If cooling is sufficiently fast, the viscosity can become so high that molecular diffusion is effectively halted, and the probability of nuclei formation becomes negligible. The sample is then said to be a glass or vitreous solid, and the process is called vitrification (Sakai, 2004). A glass is amorphous (unlike a crystal, it has no order) but has the mechanical properties of a solid. Frozen and freeze-dried plant materials behave like highly viscous metastable amorphous materials with either 'rubber' or glass' characteristics, depending on the final temperature and moisture content (Benson *et al.*, 2004).

Vitrification-based, cryopreservation protocols, which result in the elimination of most or all freezable water from samples by physical or chemical dehydration before freezing in liquid nitrogen is considered the key step for post-storage and survival (Dussert *et al.*, 2001; Engelmann, 2000b). However, the development of cryopreservation protocols using vitrification is not always straightforward especially for sensitive germplasm with complex physiology. It often requires the optimisation of chemical and thermal treatments to achieve maximal cell survival and thermal stability.

2.10 Developing Cryopreservation Protocols

In developing cryopreservation protocols, there are several approaches to vitrification which involve manipulating water/solute composition to increase cell viscosity using evaporative desiccation, osmotic dehydration and loading the cells with penetrating cryoprotectants or chemical additives.

2.10.1 Evaporative desiccation

Zygotic embryos or embryonic axes can be directly desiccated by placing them in the air current of a laminar airflow cabinet. However, more precise and reproducible desiccation conditions are achieved by placing the embryos in a stream of compressed air (Pammenter *et al.*, 1991) or in an airtight container with silica gel (Wood *et al.*, 2003) or by placing the embryos over a saturated salt solution (Dussert *et al.*, 2001; Hor *et al.*, 2005). The duration of the desiccation period varies, mainly depending on the initial water content and size of the embryos (Fang *et al.*, 2004). Usually, the water content ensuring maximal survival after freezing is around 15-20% (fresh weight basis). Desiccation must be sufficient to ensure survival after freezing, but not so extreme so as to induce desiccation injury (Wesley-Smith *et al.*, 2001). Zygotic embryos of the following tropical species have been successfully cryopreserved after direct desiccation, *Artocarpus heterophyllus* (Krishnapillay, 2000), *Swietenia macrophylla* (Marzalina and Normah, 2001; *Baccaurea motleyana* and *B. polyneura* (Normah and Marzalina, 1995), *Elaeis guineensis* (Engelmann *et al.*, 1995). In this thesis, two different types of evaporative desiccation (air and silica gel) were tested for cryopreservation for *Cassia siamea* seeds (Section 6.4).

2.10.2 Osmotic dehydration

For materials sensitive to direct desiccation, cryopreservation protocols combine preculture on media with cryoprotectants, followed by desiccation. Preculture treatment markedly enhances the freeze tolerance of cells and tissues (Sharma and Sharma, 2003; Chang and Reed, 2001; Cho *et al.*, 2001). A requirement for successful cryopreservation of sensitive germplasm is often the preculture phase involving culture on a medium containing osmotically active additives (generally sugars such as sucrose or glucose) followed by evaporative dehydration under a laminar air flow or by silica gel, then they are frozen rapidly in liquid nitrogen (Engelmann, 2004). Cryoprotectants such as sucrose partially dehydrate samples through osmotic effects and this increases the concentration of intracellular solutes and increases tolerance to subsequent evaporative dehydration (Sakai, 2004). High sucrose pretreatments applied

during preculture have been reported to improve survival of cryopreserved germplasm. For example, high survival was reported for embryos of oil palm (*Elaeis guineensis*) after preculture in 0.75M sucrose followed by 16 hours evaporative desiccation and rapid freezing (Dumet *et al.*, 2000a). A successful preculture procedure has also been developed for meristematic clumps of *Musa* sp. (Panis *et al.*, 2000). Sucrose preculture was tested for the first time for cryopreservation for *Sterculia cordata* zygotic embryos in this thesis (Section 7.4).

Besides the following cryoprotectants applied in preculture media (e.g. sucrose, sorbitol, mannitol, dimethyl sulfoxide, glycerol, lactose, raffinose, myo-inositol, maltose, glucose and fructose), trehalose (Figure 2.1) has also been reported as an effective cryoprotectant of many species (Wang and Haymet, 1998; Crowe *et al.*, 2004). Trehalose is a non-reducing disaccharide of glucose that is found widely in bacteria, fungi, insects and plants (Leslie *et al.*, 1995; Jorge *et al.*, 1997; Iturriaga *et al.*, 2000). Trehalose is found at particularly high concentrations in so-called anhyrobiotic organisms, which are capable of surviving extremely low water levels (Crowe *et al.*, 1998). Several higher plants have been reported to produce trehalose in response to drought stress. The sugar is produced either by the plant itself or by an associated symbiont (Ghasempour *et al* 1998; Goddijin and Van Dun, 1999; Iturriaga *et al.*, 2000).

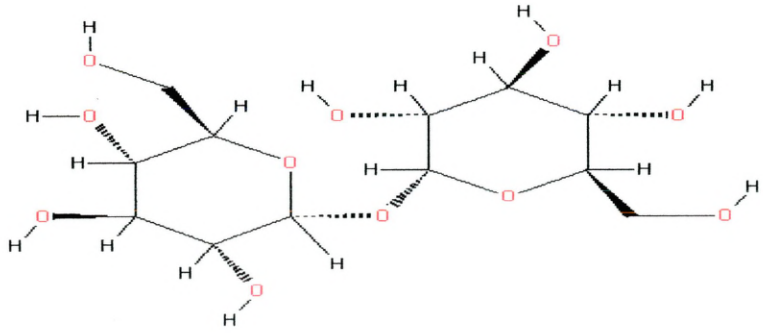


Figure 2.1 Structure of trehalose

Trehalose has been used as a cryoprotectant for many higher plants (Chen *et al.*, 1984; Bhandal *et al.*, 1985). Successful cryopreservation was demonstrated with cell cultures of *Daucus carota, Nicotiana tabacum* and *N. plumbaginifolia* using trehalose as a sole cryoprotectant (Bhandal *et al.*, 1985). It has also been reported as a cryoprotectant as well as a preculture additive for many species including *Digitali purpurea, Tabernaemontana coronaria, Plumeria rubra* and *Sterculia species* (Goldner *et al.*, 1991). However, no research has been undertaken using trehalose as a cryoprotectant for tropical tree species. The use of naturally occurring compounds like trehalose which are not toxic would be advantageous in cryopreservation studies, especially for sensitive tropical recalcitrant materials. Hence, in this thesis, the novel application of trehalose as cryoprotectant in the preculture medium for *Parkia speciosa* shoot-tips, a recalcitrant seed producing species was executed (Chapter 8).

2.10.3 Combination of evaporative and osmotic dehydration

Encapsulation-dehydration is a combination of evaporative and osmotic dehydration which is based on technologies developed for the production of artificial seeds (Redenbaugh, 1993). This technique is usually applied for the cryopreservation of apices, somatic or small embryos which are encapsulated in beads of alginate and pre-grown for a given duration in a medium with high sucrose concentration. These beads are then partially dehydrated under the air current of a laminar flow cabinet or using silica gel followed by rapid freezing into liquid nitrogen. This technique has been successfully used for the cryopreservation of the zygotic embryos of *Swietenia macrophylla* (Marzalina *et al.*, 1994), apices of citrus (Gonzalez-Arnoa *et al.*, 2000) and shoot-tips apices of many tropical species (Takagi, 2000).

2.10.4 Vitrification using chemical additives

Vitrification of cells can be achieved through the application of highly concentrated vitrification solutions. The vitrification solution sufficiently dehydrates cells without causing injury so that they can form a stable glass

along with the surrounding vitrification solution when freezing into liquid nitrogen. In addition, penetrating cryoprotective additives such as dimethylsulfoxide (DMSO) and glycerol protect cells during vitrification by increasing solute concentration and hence cell viscosity (Muldrew *et al.*, 2004). Sakai (2004) has developed Plant Vitrification Solution Two (PVS2), which is a glycerol based vitrification solution containing 30% (w/v) glycerol, 15% (w/v) ethylene glycol and 15% (w/v) dimethyl sulfoxide, prepared in the Murashige and Skoog basal medium (Murashige and Skoog, 1962) to a final concentration of 0.4 M sucrose in the cryoprotectant mixture at pH 5.8. Complete vitrification eliminates the potentially damaging effect of intra-and extra cellular crystallisation and produces high levels of cell survival (Benson, 2004). Benson *et al,* (1996b) confirmed this with the Differential Scanning Calorimetry (DSC) profiles for PVS2 treated *Ribes* apices, which showed no evidence of ice nucleation on cooling (at −10°C/min). The vitrification-based approach is suitable for complex organs, such as shoot-tips and embryos that contain a variety of cell types, each with unique requirements under conditions of freeze-induced dehydration. Successful applications of PVS2 vitrification have been reported for citrus species embryonic axes cryopreservation (Cho *et al.*, 2002) and shoot-tips of *Sechium edule* (Abdelnour-Esquivel and Engelmann, 2002). In this thesis, vitrification using chemical additives cryopreservation was conducted on *Sterculia cordata* zygotic embryos (Section 7.4).

2.10.5 Encapsulation-vitrification

Encapsulation-vitrification is a combination of encapsulation and vitrification (Hirai and Sakai, 1999). Though encapsulation and vitrification have the potential to be used individually, there are some limitations. For encapsulation, lower rates of recovery (Hirai *et al.*, 1998, Mandel *et al.*, 1996, Marzalina, 1995), and long-term lag phases or regrowth can occur after dehydration (Matsumoto *et al.*, 1995, Niino and Sakai, 1992). In PVS2 vitrification, the duration of treatment with highly concentrated vitrification solutions has to be very precisely timed in order to avoid toxicity (Sakai *et al.*, 1990). Therefore, Sakai and Matsumoto (1996), recommended an effective

combination of these techniques as an excellent solution for the cryopreservation of sensitive materials such as recalcitrant germplasm. It is a reliable, simplified and effective protocol where explants are encapsulated in alginate beads, osmo-protected and then dehydrated with a highly concentrated vitrification solution for two to three hours before rapid freezing. This technique was reported to be successful for the cryopreservation of apices of lily, wasabi, potato and chrysanthemum (Matsumoto *et al.*, 1995; Sakai and Matsumoto, 1996; Hirai *et al.*, 2000; Sakai *et al.*, 2000).

This combination method was applied for the first time for cryopreservation of *Parkia speciosa* shoot-tips in this thesis (Chapter 8). This was because this species produces recalcitrant seeds and therefore is sensitive to moisture loss and direct desiccation.

2.10.6 Cooling and warming

The cooling process involves complex phenomena and cryobiological studies have led to investigations as to what occurs during cooling of living cells and how adverse freezing phenomena can be overcome. Since water is the major component of all living cells and must be available for the chemical processes of life to occur, cellular metabolism is thought to stop when all water in the system is converted to ice (Muldrew *et al.*, 2004). However, this will be dependent upon the temperature or storage since according to Benson and Bremner (2004), free radical activity still occurs at minus 20°C and 80°C.

Ice forms at different rates during the cooling process (Mazur, 2004). During slow cooling, freezing occurs external to the cell before intracellular ice begins to form (Farrant *et al.*, 1997). As ice forms, water is effectively removed from the extracellular environment and an osmotic imbalance occurs across the cell membrane leading to water migration out of the cell. The increase in solute concentration outside the cell, as well as intracellularly, can be detrimental to cell survival (Acker and McGann, 2001). If too much water remains inside the

cell, damage due to ice crystal formation and re-crystallisation during warming can occur (Mazur, 2004).

The rate of cooling has a dramatic effect on these phenomena. Rapid cooling minimises the solute concentration effects as ice forms uniformly, but leads to more intracellular ice (Benson *et al.*, 2004). Slow cooling, on the other hand, results in a greater loss of water from the cell and less internal ice, but increases the solution effects. Cell permeability affects the rate of water loss; more permeable cells are able to tolerate rapid cooling better than less permeable cells. Mazur (2004), have postulated that ice crystal formation and solution affects both play a role in cell inactivation, and that an optimum cooling rate minimises the effect of each.

In this thesis, two different types of cooling (rapid and slow) were tested for cryopreservation of *C. siamea* seeds (Chapter 6).

The dynamics of warming is as equally important as freezing rate. To function, a frozen cell must be returned to normal temperature and must survive the accompanying warming and thawing of the ice. In the case of slowly cooled cells, the events accompanying the return to ambient temperatures are mostly a reverse of those that occurred during cooling (Mazur, 2004). However, that is not the case with rapidly cooled cells. Commonly, rapidly cooled cells fare substantially better if the subsequent warming is rapid than if it is slow. This is related to the size of the ice crystals formed during the initial cooling and the effect of warming rate on that size. Higher cooling rates form smaller internal ice crystals, and small crystals appear to be less damaging than large ones. However, if subsequent warming is slow, those small crystals can enlarge to damaging size by the process of recrystallisation (Mishima and Stanley, 1998). Different levels of warming rates were also tested for cryopreservation of *C. siamea* seeds in this thesis (Chapter 6).

2.10.7 Applications of thermal analysis

Differential Scanning Calorimetry (DSC) is being increasingly used in cold tolerance studies of both animals and plants (Wharton and Block, 1997; Pritchard and Manger, 1998). It is a powerful tool that monitors thermal transition in samples of tissues and also used to detect formation of intracellular ice crystals upon freezing and recrystallisation upon rewarming, which are the main causes of mortality during cryopreservation in liquid nitrogen (Martinez and Revilla, 1998). The main cryobiological application of DSC is to analyse the physical state of water during cooling and heating cycles to assist in the development of vitrification-based methods (Benson *et al.*, 1996a).

An important application of DSC is for the optimisation of vitrification-based protocols, particularly for storage-recalcitrant germplasm (Bachiri *et al.*, 2001). In general, DSC is used to measure water phase transitions in cryopreserved samples. The principle and practice of DSC thermal analysis is to measure heat flow differentials during water state transitions in cryoprotected samples as compared to the thermal controls. Data are recorded as a function of time and temperature. Pretreated samples (of known weight) are sealed in an air-tight aluminium alloy pan and placed inside the DSC chamber alongside an unloaded reference pan, both are simultaneously cooled or warmed in parallel at a programmable rate ($10°C.min^{-1}$) to a prescribed final temperature. Differences in heat flow between the two pans is measured during cooling and heating and the instrument software plots this function against temperature or time to produce a heat differential thermogram.

Thermal events, such as the latent heat of fusion alter the amount of heat required to maintain both pans at the same temperature, causing a change in the heat flow and this differential deflection in the thermogram is associated with thermal transitions in the sample. Transitions between liquid-ice and amorphous glassy states can be detected from heat flow data manifested as an exothermic peak during cooling and an endothermic peak during re-warming of samples. The point of change from liquid to ice is

nucleation and a liquid to a glass is the glass transition (Tg) temperature detected as deflections in heat flow. Such frozen Tg-samples behave like highly viscous metastable amorphous substances with either 'rubber' or glass' characteristics, depending on the final temperature of transition and the sample moisture content (Mazur, 2004). Thermal profiles provide critically important information about the effectiveness of cryoprotective treatments required to prevent lethal ice formation and conditions to stabilise glasses.

There are two water components during cooling that do not form ice in the supercooled state; the glass transition and the other is known as 'unfreezable water', 'unfrozen water', 'bound water' or 'osmotically inactive water' (Wolfe *et al.*, 2002). In this study, depending on its ability to participate in colligative cryoprotective processes, the water content will be characterised as either osmotically inactive or osmotically active water. Therefore, DSC can be used to detect the presence or absence of freezable water in plant tissues. This information is of value to biodiversity conservationists involved in developing cryopreservation protocols based on desiccation and vitrification techniques especially for recalcitrant seeds. Many cryopreservation researches have now incorporated DSC to examine the physical phase changes occurring during cryogenic treatments (Hor *et al.*, 2005; Dumet *et al.*, 2000b; Kim *et al.*, 2005; Dussert *et al.*, 2001; Martinez and Revilla, 1998; Vertucci *et al.*, 1991).

In this thesis, the DSC thermal analysis was applied for the first time to *P. speciosa,* a tropical recalcitrant forest tree species. This will provide an in site to the thermodynamic events that occur during cooling and subsequent rewarming and the effectiveness of the cryopreservation protocol applied.

2.10.8 Post-cryopreservation viability assessments

In cryobiology, viability is defined as the ability to survive cryogenic storage. The definition is extended to exhibit a specific function or functions, expressed as a proportion of the same function exhibited by the same sample before storage or an identical fresh sample (Berjak *et al.*, 1999). Post-

cryopreservation viability assessments depend on the material used for cryopreservation. For seeds sampled from either *in vivo* or *in vitro* environment, a germination test is usually conducted to evaluate the effect of LN storage (Wood *et al.*, 2003). In this thesis, for the orthodox seed, *C. siamea*, both *in vivo* and *in vitro* germination conditions were tested after cryopreservation (Chapter 6). For *in vitro* germination, post-cryo storage tissue culture manipulations can greatly influence the survival and regeneration capacity of cryopreserved plants. Therefore, the use of specific recovery culture medium is important for initiation of cell regrowth since its composition has been shown to significantly affect cellular injury after rewarming (Lynch, 1999). In this thesis, where zygotic embryos and shoot-tips were used for cryopreservation, *in vitro* germination condition was applied using Murasghie and Skoog medium supplemented with benzyl amino purine as growth regulator. Germination, followed by shoot and root formation and complete plant regrowth is a more reliable method for assessing field survival for plant materials. For a rapid viability assessment, vital stains such as fluorescein diacetate (FDA) and Triphenyl Tetrazolium acetate (TTC) are frequently applied (Steponkus and Lampher, 1967; Benson and Roubelakis-Angelakis, 1994). TTC reduction, is the most convenient and quickest method for multiple cell viability assessments (Sadia *et al.*, 2003). However, Ishikawa *et al* (1995), reported that the TTC assay could lead to an over-estimation of viability as shown by brome grass (*Bromus inermis*) after freezing. Ishikawa *et al* (ibid), suggested that viability assessment using TTC reduction method should be confirmed by other viability assays.

To conclude, this component of the introduction, this study will mainly concern the development of the cryogenic components of cryopreservation protocols from seed characterisation to the immediate stages of recovery and germination after cryogenic storage. Long-term cryopreservation assessments will not therefore be addressed in this particular study. However, it is important to be aware that cryopreservation imposes a series of stresses on plant material, which has the potential to induce genetic modification in regenerated plants. It is thus important to verify that the genetic stability of cryopreserved material is not altered (Harding, 2004; Dixit *et al.*, 2003; Gagliardi *et al.*, 2003). Studies

comparing the vegetative and floral development in the cryopreserved and control plant from the field did not reveal any differences in the characteristics studied on oil palm (Engelmann, 1991), potato (Mix-Wagner *et al.*, 2003) sugarcane (Gonzalez-Arnao *et al.*, 1996), banana (Cote *et al.*, 2000) and *Swietenia macrophylla* (Harding *et al.*, 2000).

2.11 Summary

This chapter has reviewed some commonly used cryopreservation techniques which involve many factors such as desiccation (method and duration), cooling and warming rates and many other cryoprotective factors. Each of these factors may take many values for example, different durations of desiccation. In this thesis, planned experiments are designed to determine which factors significantly affect the post-cryo response variables such as germination, shoot to root ratio and dry weights of the seedlings. In particular, the combination of values of significant factors that optimise the response variables are identified. In the next chapter, approaches to designing such experiments will be reviewed. Since tropical rain forest seeds are scarce and need to be cryopreserved soon after collection, the experiments must be performed quickly and with as few seeds as possible. Hence, the next chapter will also show how to reduce the size of an experiment without losing important information.

CHAPTER 3

INTRODUCTION TO FACTORIAL EXPERIMENTS

3.1 Introduction

The importance of the rapid and efficient optimisation of cryopreservation protocols for tropical tree seed germplasm was highlighted in Chapter two. The design of experiments that reduce the amount of germplasm required to be sacrificed and rapidly screen cryogenic protocols would be an advantage. This chapter introduces briefly the techniques for designing full factorial experiments at two levels and analysing it using ANOVA. A more comprehensive review of these topics is given in Appendix 1.

In an experiment, purposeful changes are made to independent variables or factors for which the resulting behaviour of the response variable is observed (Box *et al.*, 1978). The experimental units or materials are assigned to different groups at random, which then are given different treatments. The groups are then compared on some response variable to determine the effects of the treatments. Experiments are performed mainly for two purposes; first to identify important factors affecting the response variables and secondly, to optimise the response variables (Wu and Hamada, 2000).

3.2 Example of Full Factorial Experiment At Two-levels

An example of a full factorial experiment with three factors at two levels (coded as ± 1) is given below. A cryopreservation experiment is conducted where seed biomass was assessed after storage in liquid nitrogen after one of two desiccation times with one of two different cooling and two warming rates (Table 3.1).

The factors and levels are:

A: desiccation time (-1 = 2hr, 1 = 4hr)

B: cooling rate (-1 = rapid cooling, 1 = slow cooling)

C: warming rate (-1 = rapid warming, 1 = slow warming)

Table 3.1 Full factorial experimental layout for the example experiment

Run	A (Desiccation time)	B (Cooling rate)	C (Warming rate)	Biomass (g)
1	-1	-1	-1	58
2	1	-1	-1	69
3	-1	1	-1	51
4	1	1	-1	65
5	-1	-1	1	50
6	1	-1	1	81
7	-1	1	1	45
8	1	1	1	78

The factors' main effects are illustrated by the plots produce by the statistical package Minitab® as shown in Figure 3.1. This shows that factors A has a larger effect than factors B and C.

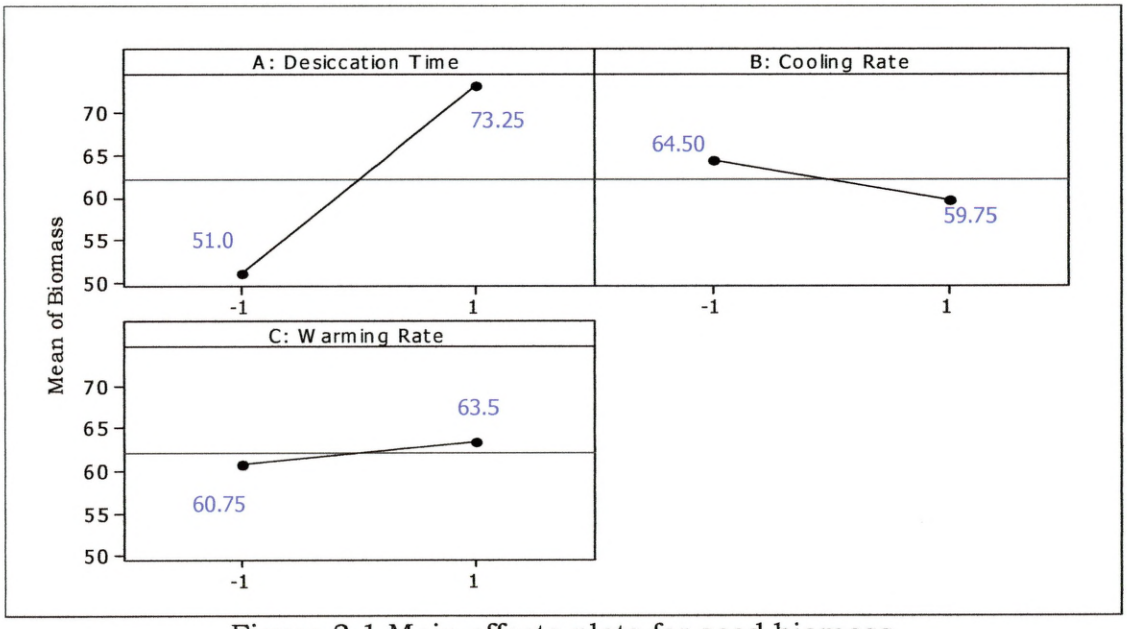


Figure 3.1 Main effects plots for seed biomass

"An interaction effect is the joint effect of two or more independent factors on the response variable" (Box *et al.*, 1978). When considering the interaction between two factors, the term two-way interactions are used in this thesis. Figure 3.2 shows the Minitab output of the two-way interaction plot for factors A, B and C.

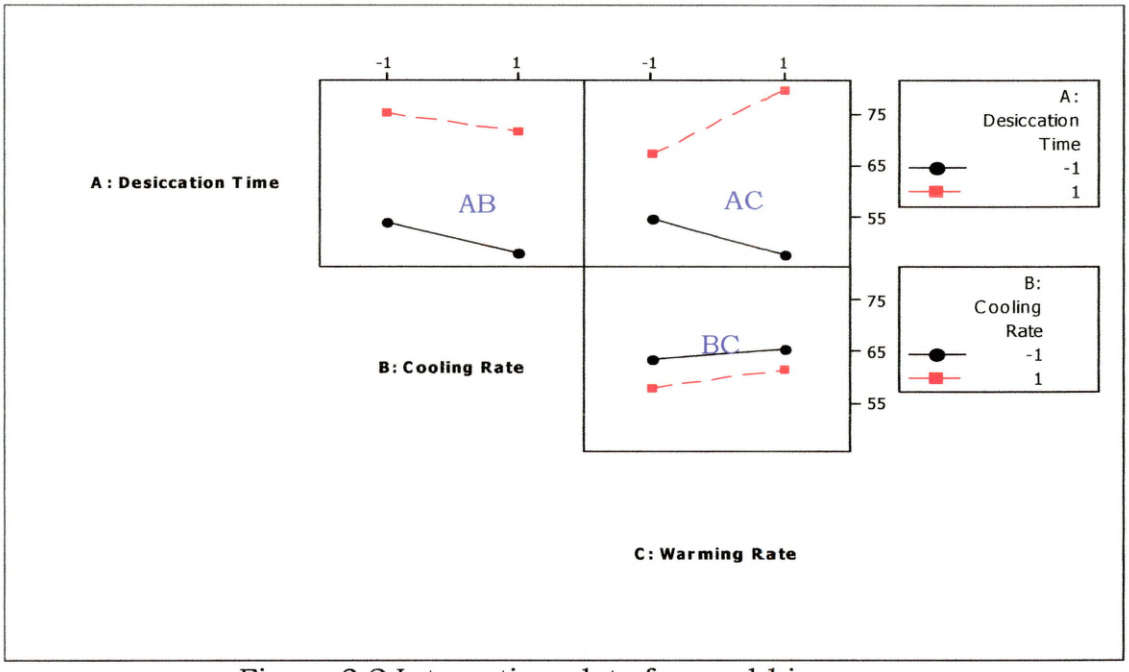


Figure 3.2 Interaction plots for seed biomass

When there is no interaction, the main effect of one factor does not change across levels of the other factor(s). Hence, the lines in the above plot are approximately parallel. This observation is noted for the AB and BC interactions. However, the main effect of factor A changes across levels of factors C and vice versa. Table 3.2a shows that A and AC are significant at the 5% level.

Table 3.2a Estimated Effects and Coefficients for seed biomass

Term	Effect	Coef	SE Coef	T	P
Constant		58.625	1.531	38.29	0.000
A	20.250	10.125	1.531	6.61	0.000
B	-4.500	-2.250	1.531	-1.47	0.180
C	-1.000	-0.500	1.531	-0.33	0.752
A*B	-1.000	-0.500	1.531	-0.33	0.752
A*C	9.000	4.500	1.531	2.94	0.019
B*C	-0.750	-0.375	1.531	-0.24	0.813
A*B*C	-0.250	-0.125	1.531	-0.08	0.937

The model for above analysis is;

Biomass = 58.625 +10.125A –2.25B –0.5C –0.5AB +4.5AC –0.375BC – 0.125ABC

ANOVA can be used to test for the significance of all main effects and interactions as shown in Table 3.2b. Note that ANOVA has a set of

assumptions which should be tested. These are summerised in Appendix 1 (Section A1.4.4.1).

Table 3.2b Second part of Analysis of Variance table for seed biomass

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Main Effects	3	1725.25	1725.25	575.083	15.34	0.001
2-Way Interactions	3	330.25	330.25	110.083	2.94	0.099
3-Way Interactions	1	0.25	0.25	0.250	0.01	0.937
Residual Error	8	300.00	300.00	37.500		
Total	15	2355.75				

In this thesis, the ANOVA results are reported as the F or t and p-values for relevant effects. Therefore, for above example, the main effect of A has a significant effect on biomass [t(8)=6.61, p=0.000]. The main effects are significant [F(3,8)=15.34, p=0.001].

Throughout this thesis, p-values are summarised into three categories. A single '*' indicates significant at 5% significance level, '**' indicates significant at 1% significance level, and a '***' indicates significant at 0.1% significance level.

3.3 Disadvantages of Full Factorial Experiments

A full factorial experiment provides a lot more information than a single factor experiment, with potentially not much more work (Montgomery, 1991). However, one limitation of the full factorial experiment is that the number of the treatment combinations rises rapidly with the number of factors (Staines *et al.*, 1999).

For example, a full factorial experiment with seven two-level factors would require 128 treatment combinations to investigate all main effects and interactions. This large number of samples can also be highly labour and cost intensive. This could be a problem in a cryopreservation protocol development experiment with tropical seeds, where experiments need to be conducted rapidly using scarce resources. A more economical approach is to

use a carefully selected fraction of the full factorial design that allows the main effects and two-way interaction effects to be estimated.

However, the reduction in data collected comes with a price, since not all interactions can be estimated, though in many cases estimating higher order interaction is of dubious value. According to the 'hierarchical ordering principle', main effects tend to dominate two-way interactions, and these in turn dominate higher-way interaction effects. This suggests that three and higher order interactions can be routinely ignored (Wu and Hamada, 2000). This assumption is used throughout this thesis.

Selection of the particular fraction to be included in the experiment, which will allow the investigation of desired main effects and/or interactions must be made with considerable care. Refer to Appendix 1 (Sections A1.5, A1.6 and A1.7) for a review of this topic.

3.4 Summary

Full factorial experiments at two-levels are the simplest experiments but the disadvantage with this design is that the number of treatment combination increases rapidly with the number of factors. Fractional factorial experiments have a smaller number of treatment combinations but they need to be selected with care to avoid aliasing important effects.

In all factorial experimental designs, as the number of factors and levels increase the experimental designs become more complicated and more difficult to analyse and interpret the data. It is therefore an advantage to have a standard method to design and analyse experiments. The next chapter will review one such approach, the Taguchi technique for designing and analysing experiments.

CHAPTER 4

INTRODUCTION TO THE TAGUCHI METHOD

4.1 Introduction

Chapter three reviewed factorial experiments and especially how fractionally replicated experiments could assist in cryopreservation protocol development experiments. However, as shown in Chapter three, the construction of fractional factorial designs is very complicated. Hence, this chapter will review the novel application of Taguchi methods to standardise the design of experiments to aid tropical germplasm cryopreservation. This chapter will highlight some special properties of Taguchi methodology and the differences between the design and analysis of factorial and Taguchi designed experiments.

4.2 The Taguchi Method

Taguchi methodology has been an important factor for Japan's post war success in electronics, automobiles, photography and other industries (Roy, 2001). Since the 1980s when it came into western industries, it has extended to many fields, especially engineering, business and management and helped to achieve more effective and robust solutions to quality control (Unal and Dean 1991). However, it is not currently applied widely in biological fields. This study is the first application of Taguchi methods to cryopreservation studies.

Taguchi's robust design is a statistical technique for standardising the design of experiments (Taguchi, 1986). It provides a method for creating fractional factorial design using 'orthogonal arrays' using a small number of observations (Refer Section 4.3.1). The prime motivation behind the Taguchi method is to achieve a reduced variation (also known as robust design), defined by the consistency of performance. This can be done by moving the mean performance to the nominal or target value as well as by reducing variations around the target. Taguchi models the departures that may occur from this target value with a loss function (Ross, 1988). The loss refers to the cost that is incurred by the society when a result obtained from an experiment is different from a nominal or target. Taguchi uses a quadratic loss function of the form:

$$L(y) = k(y-Y_0)^2$$

Where

L(y)= loss for response variable Y

k= constant

y= observed value of response variable Y

Y_0 = target value

Taguchi techniques can be adapted to optimise a response variable depending whether it needs to achieve a target value or be as small as possible or be a large as possible. The Taguchi technique, is therefore, focused on reducing variation, attaining the target value or quality and increasing consistency of a response variable, which is not addressed in the classical factorial design of experiments.

Experiments designed using Taguchi Methods are usually conducted in two phases. The first, involves running a fractional factorial experiment with each factor usually at two levels to produce a mathematical model of the results (Wu and Hamada, 2000; Sankar and Thampy, 2002). The first phase experiment will potentially have a large number of factors with two levels to predict a treatment combination near the optimal of all the investigated treatment combinations. In the second phase, only significant factors around the predicted optimal (often with more than two and usually three) levels will be investigated to produce a more accurate model, which is used to predict more precisely the optimal treatment combination and behaviour around it.

Taguchi methods make several assumptions about the target system including:

1. The experimenter has prior knowledge of which factors to vary, appropriate levels to test for each, and which interactions are of interest.

2. Interactions effects involving three or more factors are seldom significant.
3. Main effects have priority over interaction effects. If the main effect of a factor is aliased with a two-way interaction, the observed effect is allocated to the main effect.
4. When an interaction effect, say between factor A and factor B, is not investigated, the effect of factor A is assumed to be the same for all levels of factor B and the effect of factor B is assumed to be the same for all levels of factor A.
5. A model produced from the fractional factorial experiment will accurately predict a treatment combination that is near the optimal in the tested region.
6. The model produced from the fractional factorial experiment could be used to predict the optimal treatment combination in a full factorial experiment without running the experiment.

4.3 The Taguchi Method Compared to Traditional Factorial Experiments

The Taguchi approach differs from the traditional approach in two ways (Staines *et al.*, 1999). The first difference in the Taguchi approach is that of experimental design. It uses orthogonal arrays, a particular set of fractional replicates of factorial designs to design experiments (Refer to Section 4.3.1). Secondly, Taguchi does not work with the mean of the response variable instead it works with a function of the loss called a signal to noise ratio (SNR) (Refer to Section 4.3.3) (Taguchi, 1986).

4.3.1 Design of experiments using orthogonal arrays

One attribute of the Taguchi method is its efficient design of experiments using orthogonal arrays (OA) (Taguchi *et al.*, 1999). An orthogonal array is a special kind of fractional factorial matrix, in which every pair of columns includes every combination of coded levels an equal number of times. Orthogonality permits a balanced comparison of levels of any factor with a reduced number of experiments.

An example of an L_4 (2^3) OA is shown in Table 4.1. The number four refers to the four observation required, the number three in (2^3) refers to the maximum number of effects that can be estimated and the two indicates that each effect has two levels. In general for single level experiments it is written as $L_a(b^c)$, where a = number of runs, b = level of each factor and c = maximum number of effects that can be estimated.

The Taguchi method uses codes '1' and '2' for two-level experiments whereas, factorial experiments use codes '-1' and '+1' or '0' and '1' to code the two different levels of a factor in a 2^k experiment as shown below.

Table 4.1 L_4 (2^3) orthogonal array

Run	**Columns**		
	C1	**C2**	**C3**
1	1	1	1
2	1	2	2
3	2	1	2
4	2	2	1

In Table 4.1, every pair of columns includes every combination of '1's and '2's an equal number of times. For example, columns C1 and C2 have the pairs 1,1; 1,2; 2,1 and 2,2. This ensures the effects can be evaluated independently of one another so that one effect does not interfere with the estimation of another effect.

Some columns in OAs represent (i.e. are aliased with) the interactions between other columns. For example, column C3 in Table 4.1, is aliased with the interaction between C1 and C2: it has a '1' when columns C1 and C2 have the same coded level and a '2' when they differ. If factors A and B are allocated to columns C1 and C2, then the two-factor interaction between A and B, denoted AB, is represented by column C3. If factor C is allocated to column C3, the effect of AB is aliased with the effect of factor C and so the effects of AB and C cannot be separated during statistical analysis of the results. If no factor is allocated to column C3, then the effect of AB is said to

be 'clear' or it is not aliased with the effect of any other factor or two-factor interaction (Wu and Hamada, 2000).

There are many orthogonal arrays available, for example $L_4(2^3)$, $L_8(2^7)$, $L_{16}(2^{15})$ (Taguchi, 1986). The experimenter can choose an appropriate OA from these published tabulated sets of OAs (e.g. Shoemaker *et al.*, 1991). The choice depends on the number of factors and number of levels for each and the interactions of interest. The OA must have at least as many columns as the total of the factors and interactions of interest. The design of experiments could be formed by mixed levels array for efficiently analysing control factors on a various number of levels (Roy, 2001). In addition, certain orthogonal arrays types are available for examining interactions (Taguchi and Konishi, 1987).

Taguchi has provided a tool to aid in the assignment of factors and interactions to OAs by using linear graphs. There are many possible linear graphs for each OA. These linear graphs indicate to which column factors may be assigned and the columns which subsequently evaluate the interaction of those factors.

For example, the OA in Table 4.2 is represented by the two linear graphs in Figure 4.1. The dot represents a column available for a two-level factor. The line joining a pair of dots indicates the column associated with the interaction of these factors. For example, Figure 4.1a shows that column C3 is aliased with the interaction between C1 and C2, that C5 is aliased with the interaction between C1 and C4, and that C6 is aliased with the interaction between C2 and C4. Figure 4.1b shows an alternative pattern.

Table 4.2 The $L_8(2^7)$ orthogonal array

Run	**C1**	**C2**	**C3**	**C4**	**C5**	**C6**	**C7**
1	1	1	1	1	1	1	1
2	1	1	1	2	2	2	2
3	1	2	2	1	1	2	2
4	1	2	2	2	2	1	1
5	2	1	2	1	2	1	2
6	2	1	2	2	1	2	1
7	2	2	1	1	2	2	1
8	2	2	1	2	1	1	2

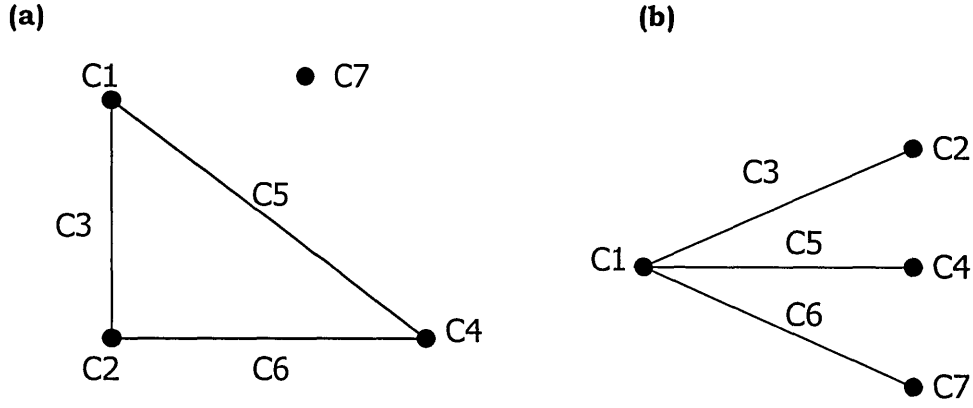


Figure 4.1. Linear graphs for $L_8(2^7)$ orthogonal array

A suitable linear graph of the OA is that which allows an allocation of factors to columns such that all specified interaction effects are clear (not aliased). For example, assume there are four factors (A, B, C and D) in an experiment and the two-way interactions of AB, AC and AD need to be investigated. The linear graph in 4.1b could be used for this purpose. If factor A is assigned to column C1, factor B needs to assigned in C2 so that AB interaction is assigned to C3. Similarly, Factor C and factor D need to be assigned in columns C4 and C7 respectively in order to investigate AC and AD interactions which will be assigned in columns C5 and C6 respectively as shown in Figure 4.2 and Table 4.3.

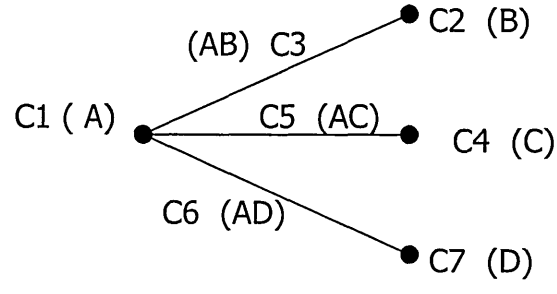


Figure 4.2. Linear graph for $L_8(2^7)$ orthogonal array

Table 4.3 The $L_8(2^7)$ orthogonal array

Run	C1 (A)	C2 (B)	C3 (AB)	C4 (C)	C5 (AC)	C6 (AD)	C7 (D)
1	1	1	1	1	1	1	1
2	1	1	1	2	2	2	2
3	1	2	2	1	1	2	2
4	1	2	2	2	2	1	1
5	2	1	2	1	2	1	2
6	2	1	2	2	1	2	1
7	2	2	1	1	2	2	1
8	2	2	1	2	1	1	2

The above design is not unique since linear graph 4.1b could also be used to design the above experiment with the specified two-way interactions. For example, if factor A is assigned to column C1, factor C to column C2 and AC interaction is allocated to column C3. If factor D to column C4, AD interaction is allocated to column C5, and if factor B is allocated to column C7, AB interaction is allocated to column C6 (Figure 4.3 and Table 4.4).

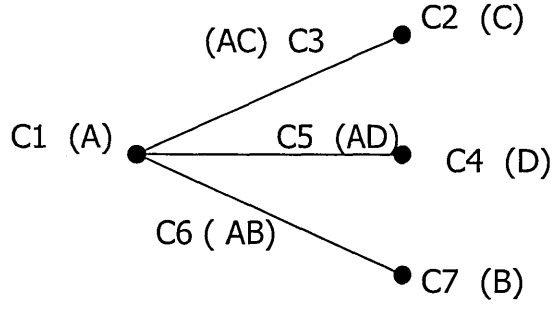


Figure 4.3 Linear graph for $L_8(2^7)$ orthogonal array

Table 4.4 The $L_8(2^7)$ orthogonal array

Run	C1 (A)	C2 (C)	C3 (AC)	C4 (D)	C5 (AD)	C6 (AB)	C7 (B)
1	1	1	1	1	1	1	1
2	1	1	1	2	2	2	2
3	1	2	2	1	1	2	2
4	1	2	2	2	2	1	1
5	2	1	2	1	2	1	2
6	2	1	2	2	1	2	1
7	2	2	1	1	2	2	1
8	2	2	1	2	1	1	2

However, it should be noted that it would not be possible to design an experiment using the linear graph in Figure 4.1b if each main effect A, B, C and the interaction AB, AC and BC were of interest. However, this is possible with linear graph in Figure 4.1a as shown in Figure 4.4.

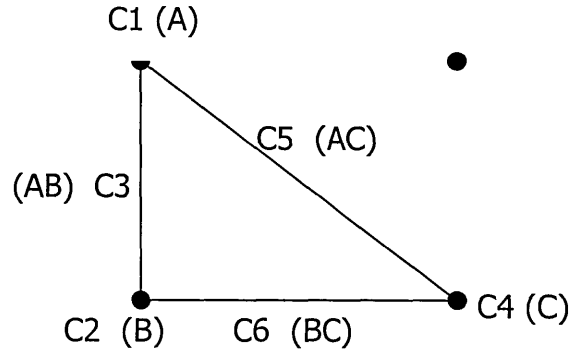


Figure 4.4 Linear graph for $L_8(2^7)$ orthogonal array

Neither linear graphs in Figure 4.1 could be used to design an experiment in which the main effects A, B, C, D and the interactions AB, AC and CD were to be investigated. If a smaller linear graph is not suitable, a larger OA with a greater number of runs is required.

Two examples are shown below on how a linear graph could assist in the choice of OA and the allocation of factors in the right columns to avoid aliasing important effects.

Consider an experiment with five factors (A, B, C, D and E) each with two levels.

Example 1: If only AB interaction is of interest

This requires an OA with at least six columns (for all the five main effects and one two-way interaction) so the $L_8(2^7)$ orthogonal array in Table 4.3 may be suitable for this design. If a linear graph is used, factors A and B should be allocated to a connected pair of dots, and no factors should be allocated to the connecting lines. The linear graph in Figure 4.1a could be used for this. Factor A could be allocated to column C1 and factor B in

column C2 and leave column C3 unused (allocated for AB interaction). This will make sure that AB interaction is not aliased with any main effects. The OA indicating the combinations to test for this experiment, is shown in Table 4.5.

Table 4.5 $L_8(2^7)$ orthogonal array with AB interaction

	C1	C2	C3	C4	C5	C6
Run	**A**	**B**	**AB**	**C**	**D**	**E**
1	1	1	1	1	1	1
2	1	1	1	2	2	2
3	1	2	2	1	1	2
4	1	2	2	2	2	1
5	2	1	2	1	2	1
6	2	1	2	2	1	2
7	2	2	1	1	2	2
8	2	2	1	2	1	1

Example 2: If all two-way interactions are of interest

There are total of five main effects and 10 two-way interactions therefore a total of 15 effects of interest. This will require an OA with at least 15 columns. Therefore, OA $L_{16}(2^{15})$ may be suitable for this experiment. The linear graph and the OA for this experiment are shown in Figure 4.5 and Table 4.6 respectively. The dots (columns C1, C2, C4, C8 and C15) are allocated for factors A, B, C, D and E respectively. The rest of the columns are allocated for the two-way interactions on the joining of the dots (e.g. C3 for AB interaction).

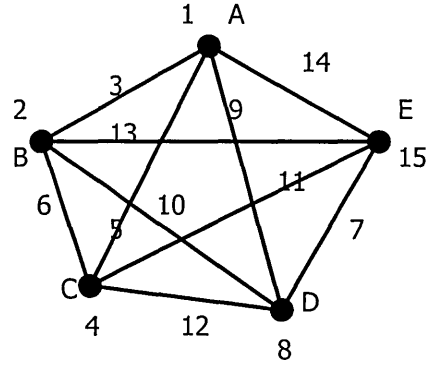


Figure 4.5 Linear graph for five factor experiment at two levels each with all the two-factor interactions

Table 4.6 $L_{16}(2^{15})$ orthogonal array

	C1	C2	C3	C4	C5	C6	C7	C8	C9	C10	C11	C12	C13	C14	C15
Run	**A**	**B**	**AB**	**C**	**AC**	**BC**	**DE**	**D**	**AD**	**BD**	**CE**	**CD**	**BE**	**AE**	**E**
1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
2	1	1	1	1	1	1	1	2	2	2	2	2	2	2	2
3	1	1	1	2	2	2	2	1	1	1	1	2	2	2	2
4	1	1	1	2	2	2	2	2	2	2	2	1	1	1	1
5	1	2	2	1	1	2	2	1	1	2	2	1	1	2	2
6	1	2	2	1	1	2	2	2	2	1	1	2	2	1	1
7	1	2	2	2	2	1	1	1	1	2	2	2	2	1	1
8	1	2	2	2	2	1	1	2	2	1	1	1	1	2	2
9	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2
10	2	1	2	1	2	1	2	2	1	2	1	2	1	2	1
11	2	1	2	2	1	2	1	1	2	1	2	2	1	2	1
12	2	1	2	2	1	2	1	2	1	2	1	1	2	1	2
13	2	2	1	1	2	2	1	1	2	2	1	1	2	2	1
14	2	2	1	1	2	2	1	2	1	1	2	2	1	1	2
15	2	2	1	2	1	1	2	1	2	2	1	2	1	1	2
16	2	2	1	2	1	1	2	2	1	1	2	1	2	2	1

This is equivalent to fractional factorial (2^{5-1}) experiment with defining contrast I=ABCDE.

4.3.2 Comparison of orthogonal arrays and factorial matrix in designing experiments

A Taguchi experiment uses an orthogonal array which is a fractional factorial matrix to design the experiments. However, both designs used different methods to allocate factors to the columns. For example, consider an experiment with three factors (A, B and C) at two levels each to investigate the main effects, three associated two-way interactions and the single three-way interaction. The full 2^3 factorial experiment is shown in Table 4.7.

Table 4.7 Factorial 2^3 experimental design

Run	**A**	**B**	**C**	**AB**	**AC**	**BC**	**ABC**
1	-1	-1	-1	1	1	1	-1
2	1	-1	-1	-1	-1	1	1
3	-1	1	-1	-1	1	-1	1
4	1	1	-1	1	-1	-1	-1
5	-1	-1	1	1	-1	-1	1
6	1	-1	1	-1	1	-1	-1
7	-1	1	1	-1	-1	1	-1
8	1	1	1	1	1	1	1
Taguchi column number	1	2	4	3	5	6	7

A Taguchi L_8 (2^3) orthogonal array is suitable for above experiment to investigate the effects up to seven two levels factors in eight runs. The use of linear graph (Figure 4.1a) could select columns one, two and four to identify the main effects A, B and C (Table 4.8).

Table 4.8 Taguchi L_8 orthogonal array

	Column Number						
Run	**C1**	**C2**	**C3**	**C4**	**C5**	**C6**	**C7**
1	1	1	1	1	1	1	1
2	1	1	1	2	2	2	2
3	1	2	2	1	1	2	2
4	1	2	2	2	2	1	1
5	2	1	2	1	2	1	2
6	2	1	2	2	1	2	1
7	2	2	1	1	2	2	1
8	2	2	1	2	1	1	2
Factorial column number	A	B	AB	C	AC	BC	ABC

For each design, each row represents a run of the experiment where they both have eight runs. Each column represents the settings of the factor at the top of the column. In the factorial design, the levels are (-1, +1); in the Taguchi design, the levels are (1, 2) each meaning the (low, high) respective levels of a factor. In the bottom row of each design is the corresponding column number for the alternative design. For example, column one in the Taguchi design corresponds to the column A in the factorial design, and vice versa. The factorial design has the runs in 'standard' or 'Yates' order (an algorithm to calculate quickly the effects from a designed experiment) (Box *et al.*, 1978). In this example, the first three columns are the factors; the first column is a series of -1's and +1's; the second column is pair of (-1,-1) then (+1,+1); and the third column is a series of (-1,-1,-1,-1) then (+1,+1,+1,+1). The interaction column elements are formed by multiplying the values in the first three columns accordingly. All seven columns are orthogonal hence all seven effects (three main effects and four interactions) can be estimated independently.

The Taguchi design has the same components as the factorial design, but in a different order. The columns for the settings of the factors are chosen according to the interactions that an experimenter think will be

important. Therefore, it requires assumptions or prior knowledge about presence of interactions before running the experiment. Linear graphs are used to determine which column to choose in the design. This example shows that the orthogonal arrays in Taguchi experiments are the same as the factorial matrix apart from the method to assign factors to columns for a full experiment.

However, both Taguchi and factorial experiment will provide different replicates for fractional experiments. An example of quarter fractional experimental design for a five factors experiment at two levels each constructed using factorial and Taguchi methods is shown in Table 4.9 and 4.10. The fractional factorial (2^{5-2}) experiment with highest resolution (III) was designed using design generator I = ABD=ACE=BCDE (Table 4.9). The Taguchi quarter experiment was designed using $L_8(2^5)$ orthogonal array (Table 4.10).

Table 4.9 Half fractional factorial design using design generator I = ABC

Run	A	B	C	D
1	-1	1	-1	-1
2	1	1	-1	1
3	1	-1	-1	-1
4	1	-1	1	-1
5	-1	-1	-1	1
6	-1	1	1	-1
7	-1	-1	1	1
8	1	1	1	1

Table 4.10 Taguchi half fractional experiment using $L_8(2^5)$ orthogonal array

Run	A	B	C	D
1	1	1	1	1
2	1	1	1	2
3	1	2	2	1
4	1	2	2	2
5	2	1	2	1
6	2	1	2	2
7	2	2	1	1
8	2	2	1	2

Note: Factorial level (-1) = Taguchi level 1 and factorial level (+1) = Taguchi level 2

Note that factorial experiment (Table 4.9) has four different runs from Taguchi experiment (Table 4.10). This example shows that different treatment combination could be selected for factorial and Taguchi fractional experiments and therefore both designs are not the same.

4.3.3 Analyzing experiments using signal to noise ratios (SNR)

The traditional method of calculating average factor effects and thereby determining the desirable factor levels (optimum condition) is to look at the simple averages or means of the results and perform ANOVA (Staines *et al.*, 1999). Although the mean is easy to calculate and interpret, it does not capture the variability of results within a trial condition.

Taguchi uses a logarithmic transformation of mean loss function called the signal-to-noise-ratio (SNR) as a single metric to identify an optimal treatment combination for a response variable in an experiment (Roy, 1990). Signal to noise ratios originally came from electrical control theory (Unal and Dean, 1991) which are based on a loss function that approximates the loss resulting from target system failing to meet the configuration goal. In general, the signal to noise ratio is defined as:

$$-10*\log_{10}(\text{mean loss function})$$

However, Taguchi proposed three signal-to-noise ratios depending on whether a response variable is needed to be minimised, maximised or to have a target value.

i) Smaller-is-better

This is usually the chosen signal to noise ratio for characteristics of which the ideal value is zero. For example, the percentage contamination in a tissue culture experiment, should be as small as possible. In this thesis, the sprouting and germination day for a seed is desired to be as small as possible.

The SNR for 'smaller is better' for a particular treatment combination is defined as;

$$\text{SNR}_{\text{Small}} = -10\log_{10}\left[\frac{\sum_i y_i^2}{N}\right]$$

where y_i (i= 1, 2, ...N) is the i^{th} observation from the particular treatment combination with a total of N replicates. If a treatment combination

consistently provides 'small' observations, then each y_i and hence the loss y_i^2 and so $\sum_i \frac{y_i^2}{N}$ will be small and the value of the SNR will be large. The optimal treatment combination is defined as that with the largest SNR.

Example of SNR_{Small} calculation from the data set below:

Table 4.11 Sprouting day for SNR_{Small} calculation

Sprouting day (SD)	**$(SD)^2$**
5	25
6	36
*	*
5	25
3	9
	$\Sigma = 95$

*=missing value

sum of loss = $\Sigma(SD)^2 = 95$, N = 5 - missing value = 5-1 = 4

Using the formula for $SNR_{Small}=-10 \log_{10} (95/4)$ = **-13.71**

ii) Larger-is-better

This criterion is usually the chosen for characteristics like survival after cryopreservation or dry weight of seedlings after cryoprotective treatments where large values of the response variable are desired. Also, when an ideal value is infinite, then the difference between measured data and ideal value is expected to be as small as possible. The SNR for 'larger is better' for a particular treatment combination is defined as;

$$SNR_{Large} = -10\log_{10}\left[\frac{\sum_i \left(\frac{1}{y_i}\right)^2}{N}\right]$$

The notations are same as in the 'smaller is better' criterions and the larger value of SNR is related to the treatment combination with the larger response variable. The optimal treatment combination is that with the larger SNR.

An example of a SNR_{Large} calculation from the data set below:

Table 4.12 Dry weight for SNR_{Large} calculation

Dry weight (DW)	(1/DW)	$(1/DW)^2$
3	0.33	0.1111
4	0.25	0.0625
6	0.17	0.02778
5	0.2	0.040
3	0.33	0.1111
		$\Sigma = 0.3525$

sum of loss = $\Sigma(1/Dw)^2 = 0.3525$, N = 5 - 0 = 5

Using the formula for SNR_{Large}=-10 $\log_{10}$ (0.3525/5) = **11.5181**

iii) Nominal-is-best

This case arises when a specified nominal or target value is most desired, and deviation in either direction is undesirable. Examples in this thesis of the applications of nominal is the best criterion are when the target value for shoot to root ratio of seedlings is one.

For 'nominal is best' criterion; $SNR_{Nominal} = -10\log_{10}\left[\frac{\sum_i (y_i - Y_o)^2}{N}\right]$,

where Y_o is the target value, and y_i and N are defined as before. The largest SNR is related to treatment combination with response variable closest to the target. Example of $SNR_{Noninal}$ calculation with target of one from the data set below:

Table 4.13 Shoot to root ratio for $SNR_{Nominal}$ calculation

Shoot to root ratio (SR)	(SR-1)	$(SR-1)^2$
0.3	-0.7	0.49
0.4	-0.6	0.36
1.1	0.1	0.01
0.9	-0.1	0.01
1.0	0	0
		$\Sigma = 0.87$

Target value = 1

sum of loss = $\Sigma(SR-1)^2 = 0.87$, N = 5 - 0 = 5

Using the formula for $SNR_{Nominal}$ = -10 $\log_{10}$ (0.87/5) = **7.59**

4.3.4 Effectiveness of mean and SNR in predicting optimal treatments

The signal to noise ratio is claimed to be more robust in predicting optimal treatments in an experiment compared to the arithmetic means (Roy, 1990). To investigate this, three treatments and seed moisture content as the response variable with a target of 10% was considered. The treatment combinations (with five replicates each) and their corresponding observed moisture contents, means and $SNR_{Nominal}$ are listed in Table 4.14.

Table 4.14 Mean and $SNR_{Nominal}$ moisture content day for various treatments

Treatments	R 1	R 2	R 3	R 4	R 5	Mean	$SNR_{Nominal}$
1	10	9	8	9	11	9.4	-1.46
2	1	1	1	24	23	10	-20.85
3	10	10	10	9	11	10	3.98

R = replicate

For the SNR calculation, the nominal is best criterion was used since a target of 10% moisture content is desirable. The calculation for the first $SNR_{Nominal}$ for treatment 1 is shown below:

$SNR_{Nominal} = -10 \log_{10} ((10-10)^2 + (9-10)^2 + (8-10)^2 + (9-10)^2) + (11-10)^2/ 5)$

$= -10 \log_{10} ((0 + 1 + 4 + 1 + 1) / 5) = -10 \log_{10} (1.4) = -10 (0.146) =$ **-1.46**

Using the mean to identify the optimum treatment will result in choosing treatments two and three since they have the mean of 10. Note that though treatment two has the mean of 10, there are three very small observations and two very large observations. Using the mean will not differentiate treatments two and three since they and both have same values. However, using $SNR_{Nominal}$ will select treatment three with the largest value and the observations are closely grouped around the target of 10. Hence, SNR is more sensitive and robust in finding the optimal treatment. Any small variation within the observed values will affect the SNR values considerably. This is shown in treatment one, where the mean differ from the target only about 0.6% but the difference in the $SNR_{Nominal}$ between treatment one and treatment three (optimal) is around 5.4 unit. For a fixed mean, SNR will be maximum when all the observed values are equal.

4.3.5 Determination of significant effects and identification of optimal treatment combination

In Taguchi experiments, the SNR approach is used as the response variable. As in factorial experiments, the same technique (ANOVA) could be used to determine which factors or interactions significantly affect the SNR. Main effects and interaction effects plots could also be produced for the SNRs.

For example, consider a three factor experiment with two-levels each and biomass as the response variable:

1. A (desiccation time: level 1= 2hr, level 2 = 4hr)
2. B (cooling rate: level 1= rapid cooling, level 2 = slow cooling)
3. C (duration of PVS2 exposure: level 1= 30 minute, level 2 = 60 minute)

The experiment is designed using $L_8(2^7)$ orthogonal array (Table 4.15). Assume that all main effects and two-way interactions are to be investigated for this experiment.

Table 4.15 Taguchi example experiment for determination of significant effects

Run	A	B	C	Biomass (Mean)	SNR_{Large} Biomass
1	1	1	1	56.00	34.96
2	1	1	2	940.00	39.46
3	2	2	1	5.62	15.00
4	2	2	2	3.16	25.00
5	1	2	1	60.40	20.00
6	1	2	2	40.52	32.47
7	2	1	1	814.15	38.59
8	2	1	2	11.22	21.00

Since the response variable is biomass the 'larger is better' criterion was used to calculate the SNR. The main effects plots for the SNR_{Large} of this experiment indicate that factors A and C have larger effects, than factor B (Figure 4.6).

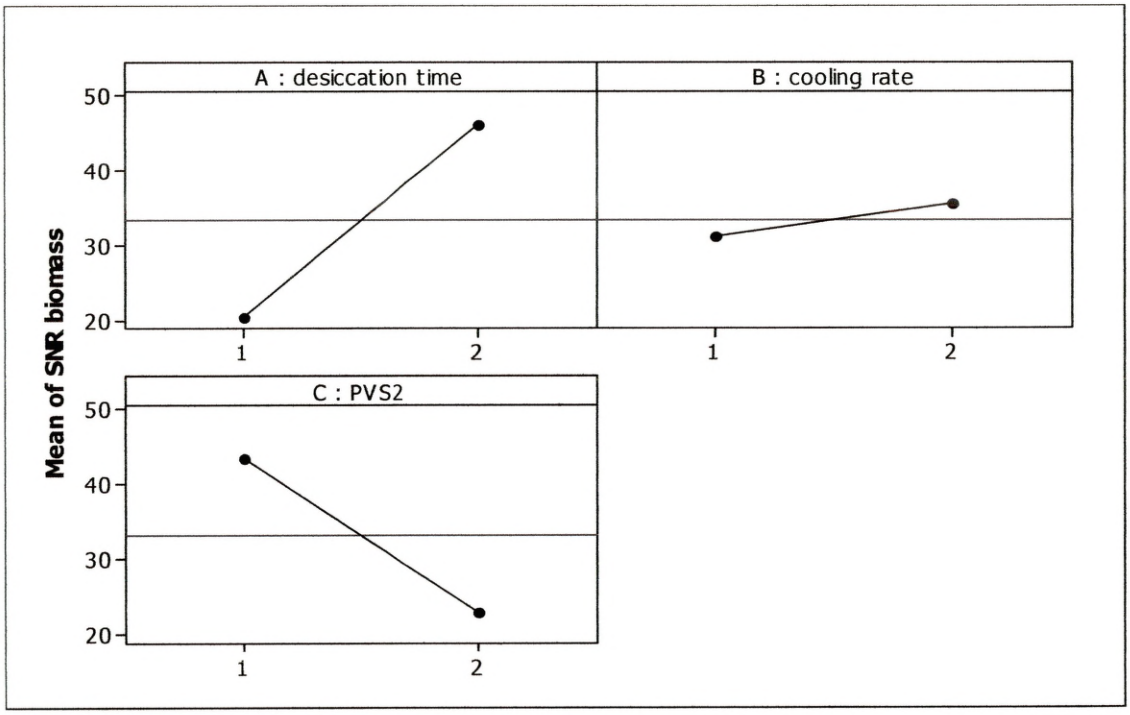


Figure 4.6 Main effects plot for SNR biomass

The AB and AC interaction plots show approximately parallel lines indicating small or no interaction effects. However, the BC interaction plot indicates a possible large interaction between factors B and C (Figure 4.7).

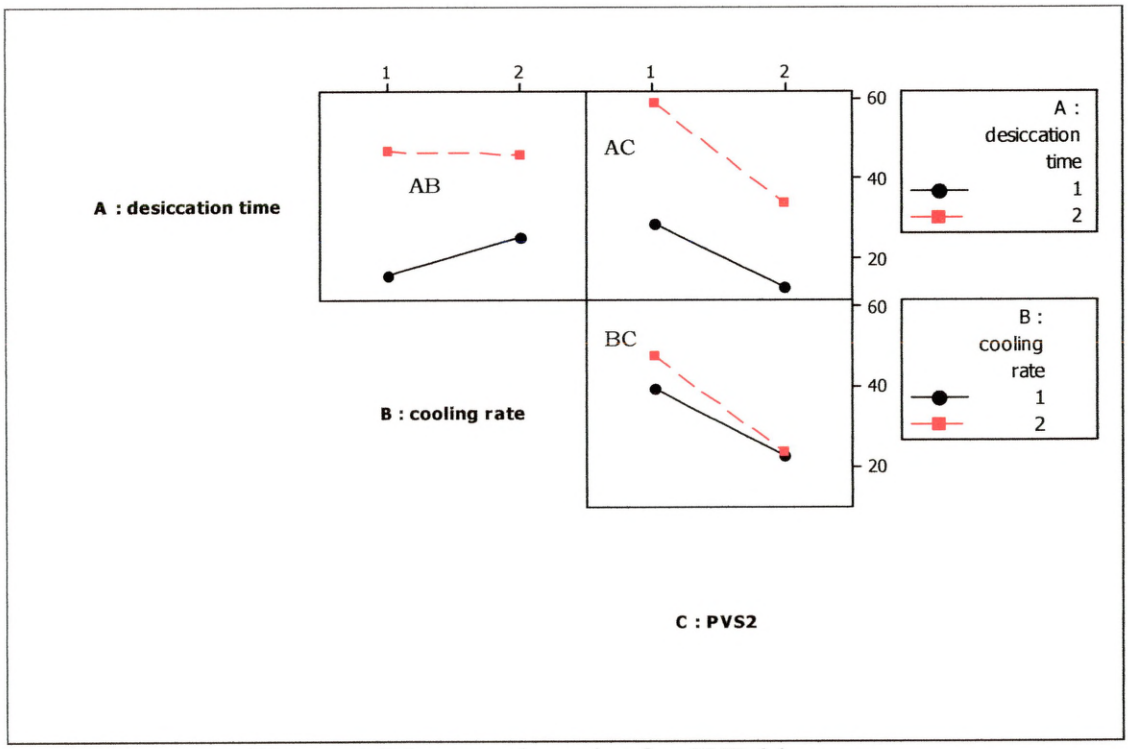


Figure 4.7 Interaction plot for SNR biomass

In Taguchi methodology, the optimal treatment combination is defined as the one which maximises the SNR. To find the optimal treatment combination, firstly, the significance of the interaction effects needed to be known only if they are thought to be important. If there is no significant interaction effect, or if they are not thought to be important then only main effects are considered. If the interactions not significant, they can be removed from the model and ANOVA is repeated but this time only with the main effects. The optimum treatment combination could be determined by referring to the 'effect' column in the ANOVA Table. The level of each factor at their optimum will be one if the sign is negative and two if the sign is positive which will give the largest SNR value.

For example, the above experiment gives the ANOVA table including all the two-way interactions as shown in Table 4.16. This shows that none of the two-way interactions are significant since each has a p-value greater than 0.05. Therefore, in the next step, ANOVA was carried out only with main effects (Table 4.17).

Table 4.16 ANOVA results for SNR Biomass for main effects A, B, C and all two-way interactions

Term	Effect	Coef	SE Coef	T	P
Constant		33.30	0.6953	47.89	0.013
A	25.79	12.90	0.6953	18.55	0.034
B	4.51	2.26	0.6953	3.25	0.190
C	-20.54	-10.27	0.6953	-14.77	0.043
A*B	-5.30	-2.65	0.6953	-3.81	0.163
A*C	-4.73	-2.37	0.6953	-3.40	0.182
B*C	-3.42	-1.71	0.6953	-2.46	0.246

R-Sq = 99.83%

Analysis of Variance for SNR biomass

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Main Effects	3	2215.56	2215.56	738.519	190.93	0.053
2-Way Interactions	3	124.31	124.31	41.436	10.71	0.220
Residual Error	1	3.87	3.87	3.868		
Total	7	2343.73				

Table 4.17 ANOVA results for SNR Biomass for factors A, B, C

Term	Effect	Coef	SE Coef	T	P
Constant		33.30	2.001	16.64	0.000
A	25.79	12.90	2.001	6.44	0.003
B	4.51	2.26	2.001	1.13	0.322
C	-20.54	-10.27	2.001	-5.13	0.007

R-Sq = 94.53%

Analysis of Variance for SNR biomas

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Main Effects	3	2215.6	2215.6	738.52	23.05	0.006
Residual Error	4	128.2	128.2	32.04		
Total	7	2343.7				

The ANOVA table above shows that factors A and C have significant effects on the SNR consistent with the main effects plot in Figure 4.3.

The model for SNR is = 33.3 + 12.9A + 2.26B – 10.27C

It was noted that effect A has positive sign whereas effect C has negative sign (effect of B can be either level 1 or 2 since it is not significant). This indicates that the best treatment combination was when factor A is at level two and factor C at level one. This is in agreement with the main effect plot in Figure 4.6. The SNR for this treatment combination can be calculated using above formula;

SNR = 33.3 + 12.9(2) + 2.26(2)– 10.27(1) = 53.35 (for effect B at level 2) and
SNR = 33.3 + 12.9(2) + 2.26(1)– 10.27(1) = 51.09 (for effect B at level 1)

The above model shows that the best treatment combination was when factor A at level two (desiccation time at hour hours) and factor C at level one (30 minutes PVS2 exposure) (Table 4.15).

In this thesis, all Taguchi experiments were analysed using ANOVA with SNR as the response variable to find the significant main effects and two-way interactions. For all the fractionally replicated Taguchi experiments, it was not possible to calculate the SNR for excluded treatment combinations

since only a fraction of the full experiment was carried out. It is possible that the optimal combination is a treatment for which the SNR is not available. To identify the optimum treatment combination a stepwise multiple regression analysis (Refer Chapter Five) was conducted to model the SNRs for all the treatment combinations. The treatment combination with highest estimates SNR is then chosen as the optimal treatment combination.

4.3.6 Follow-up or confirmatory experiment

In many cases, as discussed above, the exact combination of levels determined to be optimum may not have been one of the original test runs in the experimental design array. In such a case, it is important to perform at least one more test, with each factor set at its optimum level, to see if the result is indeed an optimised result. Such an experiment is called a confirmatory experiment. Follow-up or confirmatory experiments also could be used to determine the optimum value of the response variable more precisely. For example, in the above sample experiment, follow-up experiments can be based on the results that:

- Factor B has no significant effect on the Biomass SNR; hence it does not need to be included in the follow-up experiment.

- Factor A could be tested at levels around the optimal (four hour). Hence, for example, levels for factor A could be; 1= 3.5hr, 2 = 4.0 hr and 3 = 4.5 hr.

- An additional level to factor C could be added by including level 1= 20, level 2 = 30 and level 3 = 40 minutes PVS2 treatment, which will investigate this factor more closely.

Hence, the follow up experiment for above will only consist of factors A and C both at three levels. This follow-up experiment is simpler to set up and less costly because there are now only two factors to be varied. Adding a third level to these factors will allow investigation of a possible quadratic effect, which could be useful in drawing conclusions about these factors. A main effects plot could be used to investigate the quadratic effects. For the confirmatory experiment, the data is shown in Table 4.18 and the main effects plots are shown in Figure 4.8.

Table 4.18 Data set for confirmatory experiment

Run	A	C	SNR Biomass
1	1	1	30
2	1	2	50
3	1	3	35
4	2	1	94
5	2	2	66
6	2	3	70
7	3	1	29
8	3	2	54
9	3	3	34

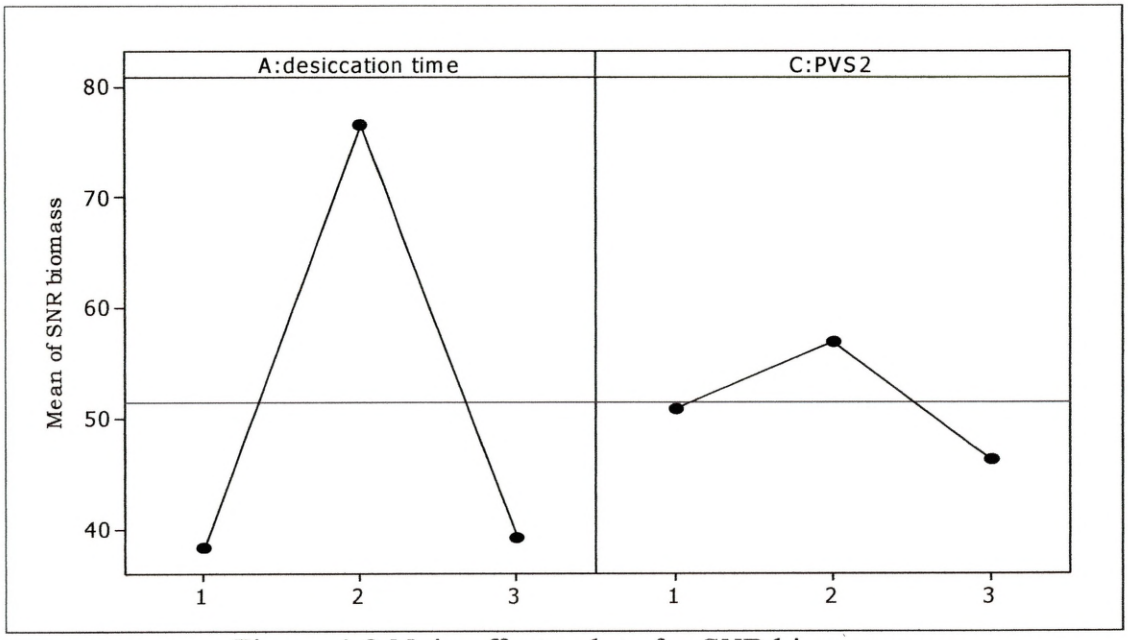


Figure 4.8 Main effects plots for SNR biomass

A response surface method could be used to investigate further the quadratic effects of the factors (Chandrasena *et al.*, 1997). Response surface methods are used to examine the relationship between one or more response variables and a set of quantitative experimental variables or factors. These methods are often employed after screening experiments where the important few factors have been identified and to find the factor settings that optimize the response. Designs of this type are usually chosen when curvature in the response surface is suspected.

Response surface methods may be employed to:

- Find factor settings that produce the 'best' response
- Find factor settings that satisfy experimental specifications or procedures
- Identify new factors or conditions that produce demonstrated improvement in the value of a response variable over the value achieved by current or ongoing protocols
- Model a relationship between the quantitative factors and the response variable

A quadratic response surface plot for factors A and C is displayed in Figure 4.9. This figure shows that the optimum levels for both factors are when they are at level two where the SNR has the maximum value. For example, SNR biomass was greater when factor A is at level two but decline when it changes to levels one and three. This was also true for factor C.

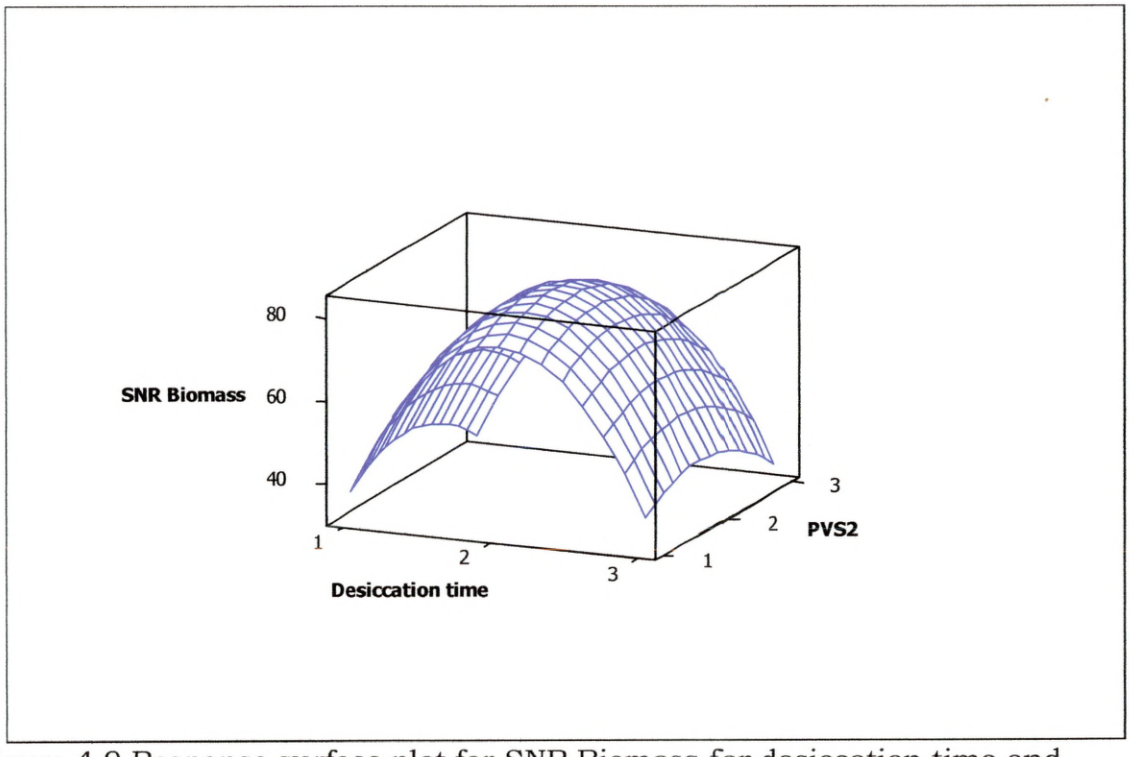


Figure 4.9 Response surface plot for SNR Biomass for desiccation time and PVS2 treatment

4.4 Application of The Taguchi Method in This Thesis

In this thesis, Taguchi methodology was applied in the following procedures:

- Full and fractional Taguchi experiments were designed using orthogonal arrays to investigate all main effects and desired two-way interactions.

- Signal to noise ratios were used as variables and compared to means. The SNRs were specifically designed to meet certain experiments' criteria. Different SNRs are suitable for different problems. For example, in Section 6.3, the response variable 'sprouting day' is desired to be a small number, 'smaller-is-better' criterion is used to calculate their SNRs.

- The results of the Taguchi experiments were analysed by using analysis of variance approach (ANOVA) to determine the significant effects. It allows for a better quantitative differentiation among the factors based on their significance.

4.5 Summary

This chapter reviewed the Taguchi methodologies for designing experiments using orthogonal arrays and linear graphs. In addition, analysing experiments using signal to noise ratios as compared to the arithmetic mean was considered. This chapter highlighted the robustness of Taguchi method in finding significant effects. This in conjunction with suitable orthogonal arrays could assist in designing small-scale experiments rapidly and finding the important effects robustly, especially in the context for tropical rainforest germplasm cryopreservation protocol development experiments. Having efficiently designed the experiments (either using traditional factorial or Taguchi methods), the next important step is to analyse them accordingly to obtain the desired information about main effects and interactions. Besides the commonly used ANOVA, regression analysis was also used in this thesis for data analysis. The next chapter, therefore, will review different types of regression analyses used in this thesis.

CHAPTER 5

REGRESSION ANALYSIS

5.1 Introduction

Chapters three and four reviewed factorial and Taguchi experiments and their analyses using ANOVA. This chapter summerises different regression and correlation analyses used in this thesis. A more comprehensive review is given in Appendix 2.

5.2 Regression Analysis

5.2.1 Simple linear regression

Simple linear regression is used to predict values of one variable, given values of another variable (e.g. if a seedling's height is to be predicted from its seed weight) (Draper and Smith, 1998). Consider a sample of ten seeds for which their dry weights and their seedlings heights are shown in Table 5.1.

Table 5.1 Data set for seed weight and seedling height

Seed weight (g)	Seedling height (cm)
3.3	16
7.1	18
13.2	34
10.2	36
9.3	30
6.4	19
4.2	15
5.1	13
12.2	38
11.6	30

The fitted regression line plot and the ANOVA table for regression analysis for the above data are shown in Figure 5.1 and Table 5.2 respectively.

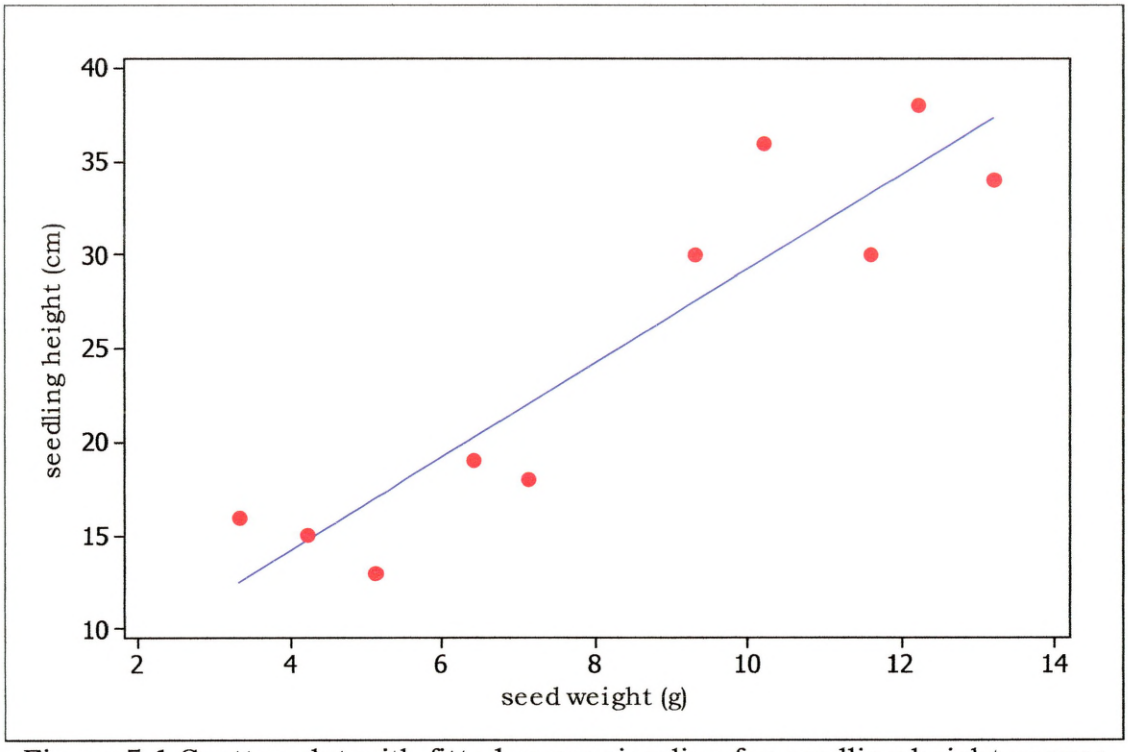


Figure 5.1 Scatter plot with fitted regression line for seedling height versus seed weight

The regression equation is:

seedling height = 4.11 + 2.52 seed weight

Table 5.2 ANOVA table for regression analysis result for seed weight versus seedling height

Source	**DF**	**SS**	**MS**	**F**	**P**
Regression	1	708.06	708.06	46.11	0.000
Residual Error	8	122.84	15.36		
Total	9	830.90			

The regression output also shows if the model can predict one variable based on another variable by the significance level of the overall F of the model from the ANOVA table. In this example, seed weight has a significant linear regression relationship with seedling height at the 5% significance level ($F(1,8) = 46.11$, $p = 0.000$). As with ANOVA, there are assumptions in regression analysis which need to be tested. These are considered in Appendix 2 (Section A2.2.2).

5.2.3 Multiple linear regression

Multiple linear regression is an extension of simple linear regression, by including several predictor variables. The multiple regression analysis equation is of the form

$$Y_i = \beta_o + \beta_1 X_{1i} + \beta_2 X_{2i} + \beta_3 X_{3i} + \ldots\ldots + \beta_k X_{ki} + \epsilon_i$$

in the usual notation.

To continue with the previous example, assume that there is another predictor variable (seed moisture content) (Table 5.3).

Table 5.3 Data set for seed weight, moisture content and seedling height

Seed weight (g)	Seed moisture content (%)	Seedling height (cm)
3.3	78	16
7.1	62	18
13.2	46	34
10.2	89	36
9.3	64	30
6.4	74	19
4.2	81	15
5.1	92	13
12.2	64	38
11.6	68	30

Table 5.4 gives the regression analysis output for the above data.

Table 5.4 The regression analysis results

Predictor	Coef	SE Coef	T	P
Constant	-8.08	11.25	-0.72	0.496
seed weight	2.8377	0.4618	6.14	0.000
seed MC	0.1328	0.1174	1.13	0.295

For above experiment the regression model is:

seedling height = - 8.08 + 2.84 seed weight + 0.13 seed moisture content

An ANOVA gives that the F ratio [F(2,7) = 24.5, p=0.001] which shows that this model is significant. However, the regression table shows that only seed weight is a significant predictor variable (p = 0.000) of seedling height but not seed moisture content (p = 0.295). This is a disadvantage of multiple regression analysis where it includes both significant and insignificant predictor variables in the model. However, this problem could be solved by using an algorithm to identify a model with only the significant effects efficiently. These are explained in Appendix 2 (Section A2.2.4).

In this thesis, fully stepwise regression analysis was used since it comprises both forward selection and backward elimination methods in it. It was used for all the fractionally replicated experiments to find a model with all the significant effects. The regression equation is used to calculate all the fitted values for the full experiment. The best treatments for the fitted values were obtained by ranking the treatment combinations. The fitted values were also compared with the observed values from the full experiment using Spearman's rank correlation (Section A2.3.2).

5.2.3 Binary logistic regression

Binary logistic regression analysis is used when the response variable follows a binomial distribution (Sokal and Rohlf, 1995). This analysis is suitable when researchers want to analyse whether some event occurred (success) or not (failure), such as viability of a plant material after cryopreservation. This is covered in more detail in Appendix 2 (Section A2.2.5).

In this thesis, binary logistic regression analysis was used to analyse data in Section 7.4 and Chapter eight, since the response variable for these experiments (viable embryos or shoot-tips after cryopreservation) followed a binomial distribution and therefore could not be analysed using ANOVA or linear regression analysis.

5.3 Correlation Analysis

5.3.1 Product-moment linear correlation (Pearson r)

Product-moment or Pearson's correlation coefficient (r) measures the degree to which two quantitative variables are linearly associated. This is outlined in Appendix 2 (Section A2.3.1).

In some applications, the assumption for Pearson's correlation coefficient are violated. In these cases, the ranks of the experimental treatment combinations can be compared using Spearman's rank correlation to investigate if they are in agreement or not (Quinn and Keough, 2002; Refer to Appendix 2 (Section A2.3.2)).

Spearman's rank correlation analysis was used in this thesis to compare the ranks of treatment combinations for all the response variables obtained using full factorial and fractional replicates and Taguchi full and fractional experiments.

5.4 Analysis of Covariance (ANCOVA)

Analysis of Covariance (ANCOVA) is a technique that sits between analysis of variance and regression analysis (Rutherford, 2000). Analysis of covariance is used when the response variable y, in addition to being affected by the controllable treatments, is also linearly related to another continuous variable x. Refer to Appendix 2 (Section A2.4) for summary of this topic.

ANCOVA was used in Section 6.5 of this thesis, to evaluate the response variables on three different types of germination substrates where desiccation time and warming rate were selected as covariates.

5.5 Summary

This chapter concludes the reviews of the statistical and experimental design methods and applications. The thesis will now progress with the application of novel experimental designs and analysis to rainforest tree germplasm cryopreservation protocol development.

For the experiments in Chapters six and seven in this thesis, full factorial and Taguchi full experiments were conducted to compare 'mean' and 'SNR' analyses. The experiments were analysed with ANOVA to investigate main effects and two-way interactions. Treatment combinations using mean and SNR were ranked and compared using Spearman's rank correlation analysis (Quinn and Keough, 2002).

The suitable subsets of fractional and Taguchi designs were selected for each experiment conducted. Stepwise regression analysis (Draper and Smith, 1998) was used to find suitable models from these fractionally replicated experiments. The stepwise regression model was used to estimate the mean response and SNR for all treatments. The fitted values were ranked and Spearman's rank correlation analysis was used to compare these with those of the observed mean and SNR values respectively from the full experiment to justify the model. Fitted mean and SNR values were also compared using Spearman's rank correlation.

CHAPTER 6

INVESTIGATING DIFFERENT EXPERIMENTAL DESIGNS TO OPTIMISE CRYOPRESERVATION PROTOCOL DEVELOPMENT FOR TROPICAL ORTHODOX SEEDS

6.1 Introduction

Having reviewed the design and analysis of factorial and Taguchi experiments with special emphasis on fractionally replicated experiments, the thesis in this chapter will focus on the practical experiments planned and carried out on a tropical orthodox seed producing species.

Orthodox seeds are known to survive cryopreservation relatively readily (Salomao, 2002). No loss in viability was reported for *Pinus ponserosa*, *Pseudotsuga menziesi*, *Thuja plicata* and *Tsuga heterophylla* following three years of cryopreservation in liquid nitrogen (Stanwood, 1985). For this study, *Cassia siamea* Lam. (International Plant Names Index, 2004) an orthodox seed, was selected as a cryopreservation case study comparing conclusions from a full factorial experiment, fractional factorial experiments and the novel application of Taguchi experimental design to optimise the development of robust experimental strategies. This species was also selected for seed availability and ease of supply, allowing all experimental parameters to be tested and providing sufficient data for comparisons of all experimental designs and data analyses. The subject of this study has been published in Muthusamy *et al.*, (2005 (*in press*)).

6.2 Species Introduction

6.2.1 Botany

C. siamea Lam. (synonyms: *Senna siamea* Lamk., *C. florida* Vahl.; *Senna sumatrana*, Roxb.) is a non-nitrogen-fixing leguminous tree in the subfamily Caesalpinoideae of the family Leguminosae. It is an important fast growing tropical species widely used in urban forestry in Malaysia (De Padua *et al.*, 1999).

C. siamea is a medium sized evergreen tree attaining five meter height and it rarely exceeds 20 m height and 50 cm diameter (Jensen, 1995). It has a dense, evergreen, irregular, spreading crown, a crooked stem, and smooth,

grayish bark that is slightly fissured longitudinally; young branches have fine hairs. The leaves are pinnately-compound with an even leaf arrangement of 7-10 pairs of ovate-oblong leaflets seven-eight cm long and one-two cm wide (Figure 6.1). The flowers are yellow, borne in large terminal panicles that are often 30 cm long. The flowering period is long, and flowers may often be found at various seasons (Troup, 1921). The fruit is a flat pod 15-25 cm long, thickened at both sutures, containing many seeds (Gutteridge, 1997).

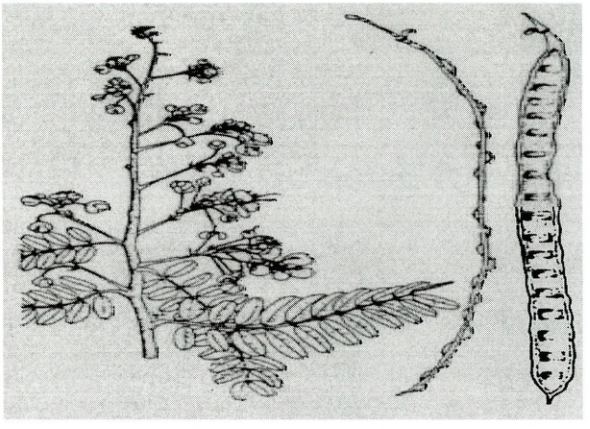


Figure 6.1. Leaves, flowers and seeds of *Cassia siamea* Lam. (source: F/FRED, 1994)

6.2.2 Ecology

C. siamea grows well in many environments, particularly in lowland tropics, having mean annual rainfall of 500-2800mm (optimum about 1000 mm), mean minimum temperature of 20°C, and mean maximum temperature of 31°C (Gutteridge, 1997). In semiarid environments with a mean annual rainfall of 500-700 mm it will grow only where its roots have access to groundwater and where the dry season does not exceed four to six months. The best growth occurs in deep, well drained, rich soils with pH 5.5-7.5. *C. siamea* tolerates well drained lateritic or limestone soils and moderately acid soils (pH 5.0) and requires full sun (Davidson, 1985).

6.2.3 Distribution

C. siamea is native to South and Southeast Asia, from Thailand and Myanmar (Brandis, 1906) to Malaysia, India, Sri Lanka, and Bangladesh (Khan and Alam, 1996). It has been cultivated worldwide and is naturalized in many locations (Gutteridge, 1997).

6.2.4 Economic importance

C. siamea wood is used for furniture, poles, small timber, and fuelwood. It is hard, with specific gravity of 0.6-0.8. The sapwood is whitish, and the heartwood is dark brown to nearly black, with stripes of dark and light. The fuelwood and charcoal are highly regarded (calorific value of 4500-4600 Kcal/kg), but the wood produces a lot of smoke (F/FRED, 1994). It has been widely planted in many Southeast Asian countries for erosion control, windbreakers, shelterbelts, fuelwood, and polewood. It is a good ornamental tree for planting along roadsides, and it is also used in alley cropping, intercropping and hedgerows. It is planted as a shade tree in cocoa, coffee, and tea plantations (F/FRED, 1994). The tree produces an extensive root system in the upper layer of the soil and, in intercropping systems, can aggressively compete for nutrients and water (Gutteridge, 1997). The leaves and seeds can be eaten by ruminants (Sahni, 1981) but are toxic to non ruminants such as pigs and poultry. The young leaves and flowers are used in curry dishes; the species is also used for the production of honey and tannins.

6.2.5 Silviculture

C. *siamea* is usually propagated by seed, and plantations are often established by direct seeding (Gutteridge, 1997). Mature pods are collected from the tree when they turn brown (Siddiqi and Ali, 1994). After collection, the pods are dried in the sun for a few days. The seeds are small and greenish-brown, and there are about 37,000-43,000 seeds per kilogram (Katoch, 1992). The seeds have a shedding moisture content of around 20-23% and are orthodox in storage category (De Padua *et al.*, 1999). Clean seeds can be stored in air-tight containers at room temperature for years

with little loss of viability. Insects that damage seed include *Caryedon lineaticollis* and *Bruchidius maculatipes*. Seed-borne fungi reported in Thailand include *Aspergillus niger* and *Curvularia pallescens* (Siddiqi and Ali, 1994). Germination is complete within 7-20 days and germination typically exceeds 70 percent. Katoch (1992) dipped the seeds in hot water for 1 minute before soaking them in cold water for 24 hours, and obtained 98 percent germination in 28 days.

6.3 Experimental procedures

Two experiments were carried out on this species. Experiment one was a full factorial screening experiment with five factors at two levels each (Section 6.4). The objective is to identify significant factors that affect the post-cryopreservation sprouting day, shoot to root ratio and seedling dry weight using both ANOVA and Taguchi analysis.

The second experiment was a confirmatory experiment for the first with three factors at three levels each (section 6.5). The selection of factors and their levels are based on the results of the first experiment providing a robust strategy for cryopreservation protocol development.

6.4 Comparing A Full Factorial Experiment With Five Factors At Two-levels, Fractional Replication and Taguchi Techniques For The Cryopreservation of *Cassia siamea* Lam. Seeds

6.4.1 Experimental overview

A cryopreservation experiment was carried out on *C. siamea* seeds and designed with five factors each at two levels as:

A: drying method (-1(1) = silica gel or +1(2)= air drying),
B: drying duration (-1(1)=4 h or +1(2)=8 h),
C: cooling rate (-1(1)=slow or +1(2)=rapid),
D: warming rate (-1(1)=slow or +1(2)=rapid) and
E: recovery media (-1(1)= soil or +1(2)= Murashige and Skoog 1962 [MS)

The –1 and +1 indicates two levels for factorial experiment and the levels in the brackets are for the Taguchi experiments for the cryogenic factors (drying at two times, cooling rate and warming rate and recovery using *in vitro* and soil-compost substrates).

6.4.2 Materials and methods

6.4.2.1 Seed material

Seedpods were collected from mother trees from the Forest Research Institute of Malaysia (FRIM) campus near Kuala Lumpur, Malaysia, and were dried in the FRIM Seed Laboratory until their pods dehisced. Seeds were washed in running water, soaked in hot water (*ca* 80 ^{o}C) for one minute (Kobmoo and Hellum, 1984), surface sterilized (10% v/v hypochlorite solution, Domestos, Lever Bros.) for 10 minutes, and rinsed once in 50% (v/v) ethanol for one minute, and then rinsed three times in sterile water.

6.4.2.2 Seed drying

For air drying, sterilized seeds were arranged in a single layer in sterile Petri dishes and placed in the air current of laminar airflow cabinet. For silica gel drying, surface sterilized seeds were placed in a desiccator containing sterile and dehydrated silica gel (VWR Int. Ltd.) and covered. The

drying period selected was four or eight hours for both methods. The room temperature and relative humidity during seed desiccation were 23 ± 2 °C and 55 ± 5% respectively.

6.4.2.3 Cooling procedures

After the required desiccation period, seeds were placed in sterile 2ml cryo-vials (Nalgene, Sigma) and cooled either rapidly by direct immersion in liquid nitrogen or slowly by using a programmable Planar Kryo 10 (Planar select, LTD, Sunbury, UK). The programme comprised a starting temperature of 25°C, a cooling rate of -1°C /min and a holding temperature of -40°C for 35 minutes followed by direct plunging in liquid nitrogen. The programme was selected based on the freezing regime of Withers and King (1980).

6.4.2.4 Warming procedures

For rapid warming, the cryo-vials were removed from liquid nitrogen and directly plunged and swirled in a water bath at 38 ± 2°C for 10-15 minutes. For slow (ambient at *ca* 25°C) warming, the cryo-vials were removed slowly from liquid nitrogen and warmed to ambient temperature for 30 minutes in a laminar airflow cabinet.

6.4.2.5 Post-cryopreservation recovery and seed germination

For *in vivo* germination, the re-warmed seeds were sown in germination boxes containing garden soil substrate as recommended by the International Seed Testing Association (ISTA), (1999). The seeds were incubated in a growth room at 25 °C with a 16 hours photoperiod. For *in vitro* germination, Murashige and Skoog (1962) medium (MS) supplemented with 30g/l sucrose (Duchefa Biochemie) and 5.5g/l Plant Agar (Duchefa Biochemie) was used. The seeds were cultured in a temperature-controlled growth room at 26 °C with a 12 hours photoperiod. The development of each seed from sprouting to germination was observed daily for 10 days.

6.4.3 Measurements and observations

6.4.3.1 Moisture content

Seed moisture content was determined on a fresh weight basis using a 103°C oven and drying for 17 hours (International Seed Testing Association (ISTA), 1999) with four replicates of 10 seeds.

6.4.3.2 Sprouting and germination

The seeds were considered sprouted when the seed coat split and the radicle emerged from the seed and were considered germinated when both radicle and plumule emerged from the seed. The development of each seed from initial sprouting to germination was observed daily.

6.4.3.3 Shoot to root ratio

After 10 days of incubation, each seedling was harvested to determine the shoot to root ratio. The seedlings were removed from the medium and the root zone was washed in running tap water to remove the soil or agar and then blotted with tissue paper. The shoot zone and the root zone of individual seedling were measured using a thread and ruler. In this study, the convention was adopted to define the best shoot to root ratio of one as developed for jackfruit, *Artocarpus heterophyllus* (Krishnapillay, 1990).

6.4.3.4 Seedling dry weight

Evaluation of seedling dry weight was carried out after measuring the shoot to root ratio. The seedlings were dried in a 100°C oven for 24 hours after which the weights were recorded.

6.4.4 Experimental design and data analysis

The full factorial (2^5) and Taguchi ($L_{32}(2^5)$) experiments were conducted using five factors each with two levels (generating 32 treatment combinations). Forty replicates (one seed is a replicate) were used per treatment combination. Half and quarter fractional factorial experiments were designed using design generator I=ABCDE (resolution V) and design generator I = ABC (resolution III) respectively. For the Taguchi half and

quarter fractional replicates, orthogonal arrays $L_{16}(2^5)$ and $L_8(2^5)$ were used. These subsets (using 16 and 8 treatments respectively) were chosen to investigate the possibility of using fractional replicated experiments (Table 6.1).

The conclusions from full and fractionally replicated experiments were compared for factorial and Taguchi designs as described in Section 5.5. For Taguchi experiments, the optimal treatment combination is that which maximises a signal to noise ratio (SNR). The response variables chosen for this case study were; sprouting day, shoot to root ratio and seedling dry weight. These illustrate the three SNRs (smaller is better, nominal value and larger is better respectively) commonly used with Taguchi experimental design.

6.4.5 Results

The average initial seed moisture content on a fresh weight basis was 20%. The moisture content decreased to 14% and 8% after 4 and 8 h drying respectively, regardless of whether air or silica gel drying method was used (Figure 6.2). Germination of the seeds after desiccation before cryopreservation was greater than 80% for all desiccation treatments (Figure 6.2). Cryopreservation was successful with *C. siamea* seeds and high germination (> 70%) was achieved for all cryoprotective treatments (Table 6.1).

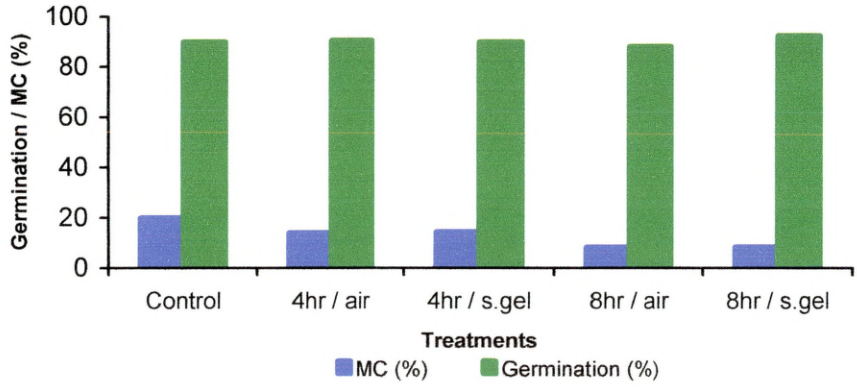


Figure 6.2. Percentage moisture content (fresh weight) and germination of *C. siamea* seeds after 4 and 8 hours of desiccation

Table 6.1. Treatment combination selected for each experimental design and the percentage germination of cryopreserved *C. siamea* seeds

Run	DM	DT	CR	WR	M	$2^5/L_{32}$	2^{5-1}	L_{16}	2^{5-2}	L_8	Germination (%)
1	Air	4h	R	R	Soil	Y			Y	Y	75.0
2	Air	4h	R	R	MS	Y	Y	Y			77.5
3	Air	4h	R	S	Soil	Y	Y	Y			75.0
4	Air	4h	R	S	MS	Y				Y	75.0
5	Air	4h	S	R	Soil	Y	Y	Y			87.5
6	Air	4h	S	R	MS	Y			Y		80.0
7	Air	4h	S	S	Soil	Y					92.5
8	Air	4h	S	S	MS	Y	Y	Y			85.0
9	Air	8h	R	R	Soil	Y	Y				87.5
10	Air	8h	R	R	MS	Y					92.5
11	Air	8h	R	S	Soil	Y		Y	Y		90.0
12	Air	8h	R	S	MS	Y	Y	Y			80.0
13	Air	8h	S	R	Soil	Y				Y	87.5
14	Air	8h	S	R	MS	Y	Y	Y			82.5
15	Air	8h	S	S	Soil	Y	Y	Y			90.0
16	Air	8h	S	S	MS	Y			Y	Y	87.5
17	S.G	4h	R	R	Soil	Y	Y	Y			70.0
18	S.G	4h	R	R	MS	Y					82.5
19	S.G	4h	R	S	Soil	Y					87.5
20	S.G	4h	R	S	MS	Y	Y	Y	Y		85.0
21	S.G	4h	S	R	Soil	Y					87.5
22	S.G	4h	S	R	MS	Y	Y	Y		Y	80.0
23	S.G	4h	S	S	Soil	Y	Y	Y	Y	Y	80.0
24	S.G	4h	S	S	MS	Y					85.0
25	S.G	8h	R	R	Soil	Y					82.5
26	S.G	8h	R	R	MS	Y	Y	Y	Y	Y	80.0
27	S.G	8h	R	S	Soil	Y	Y	Y		Y	90.0
28	S.G	8h	R	S	MS	Y					87.5
29	S.G	8h	S	R	Soil	Y	Y	Y	Y		90.0
30	S.G	8h	S	R	MS	Y					87.5
31	S.G	8h	S	S	Soil	Y					90.0
32	S.G	8h	S	S	MS	Y	Y	Y			90.0

Index: T=Treatment, DM=drying method, DT=drying time, CR=cooling rate, WR=warming rate, M=medium, S.G=silica gel, R=rapid, S=slow, MS=Murashige and Skoog (1962), Y=selected treatments

6.4.5.1 Comparisons of ANOVA and SNR for the full experiment

6.4.5.1.1 Sprouting day

Five way ANOVA including all main effects and two-way interactions showed that drying time [F(1,16)=15.58, p=0.001] and medium [F(1,16)=108.10, p=0.000] have a significant effect on sprouting day (Figure 6.3). A smaller mean, regarded as advantageous, was observed with seeds dried for 4h in soil. A significant two-way interaction was noted for the drying method with cooling rate [F(1,16)=6.80, p=0.019] (Figure 6.4).

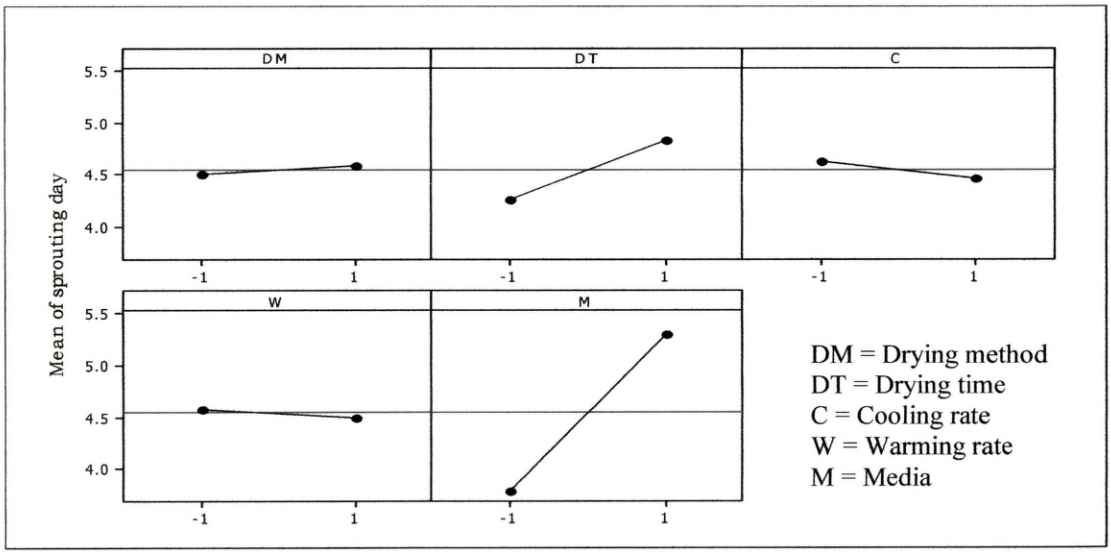


Figure 6.3 Main effect plots for sprouting day

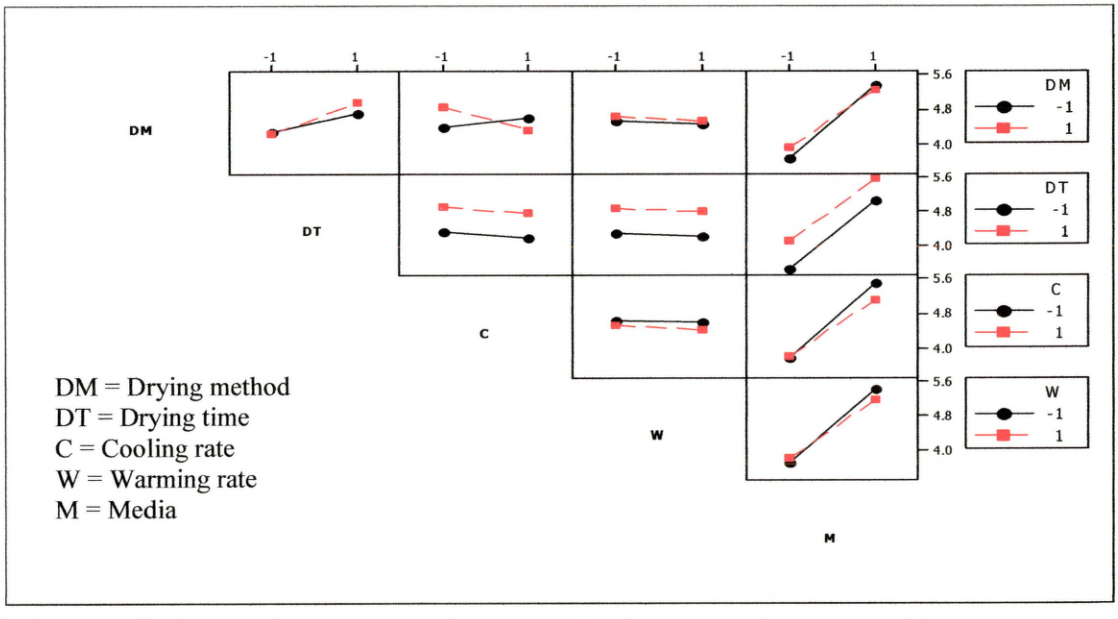


Figure 6.4 Interaction plot for sprouting day

The Taguchi analysis gave the same significant effects, which are summarised in Table 6.8. Spearman's rank correlation coefficient (r_s), between the mean and SNR_{Small} was equal to one, showing that the treatments' ranks are the same for both mean and SNR. Therefore, the conclusions obtained from ANOVA (mean) and Taguchi (SNR) for the full experiments are the same. Table 6.2 shows the five best treatments selected for the smallest sprouting day using the mean, their corresponding SNR_{Small} and rank.

Table 6.2: Five best treatments selected for the smallest mean sprouting day their corresponding SNR_{Small} and rank in the full experiment

Drying method	**Cooling rate**	**Warming rate**	**Mean (day)**	**Rank Mean**	**SNR**	**Rank SNR**
Air	Slow	Rapid	2.97	1	-9.74	1
Air	Rapid	Rapid	3.07	2	-9.91	2
S.gel	Rapid	Slow	3.29	3	-10.46	3
S.gel	Slow	Rapid	3.42	4	-10.83	4
Air	Rapid	Slow	3.43	5	-10.90	5

Note: For all the treatments selected above, the seeds were dried for 4 h and germinated in soil substrate

6.4.5.1.2 Shoot to root ratio

Shoot to root ratio equal to one is regarded as optimum for this variable. The medium [F(1,16)=168.49, p=0.000] had a significant effect on the shoot to root ratio, with seeds germinated in soil having a shoot to root ratio closer to one. A significant two-way interaction was noted between drying method and cooling rate [F(1,16)=9.35, p=0.008].

For the Taguchi's $SNR_{Nominal}$, medium [t(16)=-14.95, p=0.000] and cooling rate [t(16)=-2.94, p=0.010] were significant. The negative effects for medium and cooling rate indicate soil medium and a rapid cooling rate are preferred respectively. This analysis also showed significant two-way interactions between cooling rate and media, drying time and media and also between drying time and warming rate (Table 6.8). Spearman's rank correlation coefficient between mean closest to one and $SNR_{Nominal}$ was r_s=0.950, ($p<0.05$). This shows a significant but not exact agreement for both

methodologies. Table 6.3 shows that the treatment combination with mean closest to one is not that with the largest SNR.

Table 6.3: Five best treatments selected for the mean shoot to root ratio closer to one, their corresponding $SNR_{Nominal}$ and rank in the full experiment

Drying method	**Drying time**	**Cooling rate**	**Warming rate**	**Mean**	**Rank Mean**	**SNR**	**Rank SNR**
S.gel	4hr	Rapid	Rapid	0.9832	1	11.548	6
S.gel	4hr	Slow	Rapid	1.0437	2	15.026	1
S.gel	8hr	Rapid	Slow	0.9308	3	10.499	10
Air	8hr	Rapid	Slow	0.8897	4	11.617	5
Air	4hr	Slow	Rapid	0.8840	5	14.864	2

Note: All seed were germinated in soil substrate

A further investigation was carried out to explore the differences in agreement between these two measures. Figure 6.5 shows the dot plot the shoot to root ratios for the treatment combinations with five means closest to one. The data values for the treatment with mean closest to one (rank six for SNR) are not closely distributed around the target value of one (Figure 6.5). The treatment with mean second closest to one had a larger $SNR_{Nominal}$ since these data are more closely grouped around one. For the third and fourth ranked mean values, the dot plot shows that these data are not distributed close to the target. The treatment with fifth closest mean to one, have data which are more closely grouped close to the target value. This data set is ranked second highest for $SNR_{Nominal}$. This shows that for this response variable, the results obtained by the SNR is more robust and are not unduly influenced by a few observations.

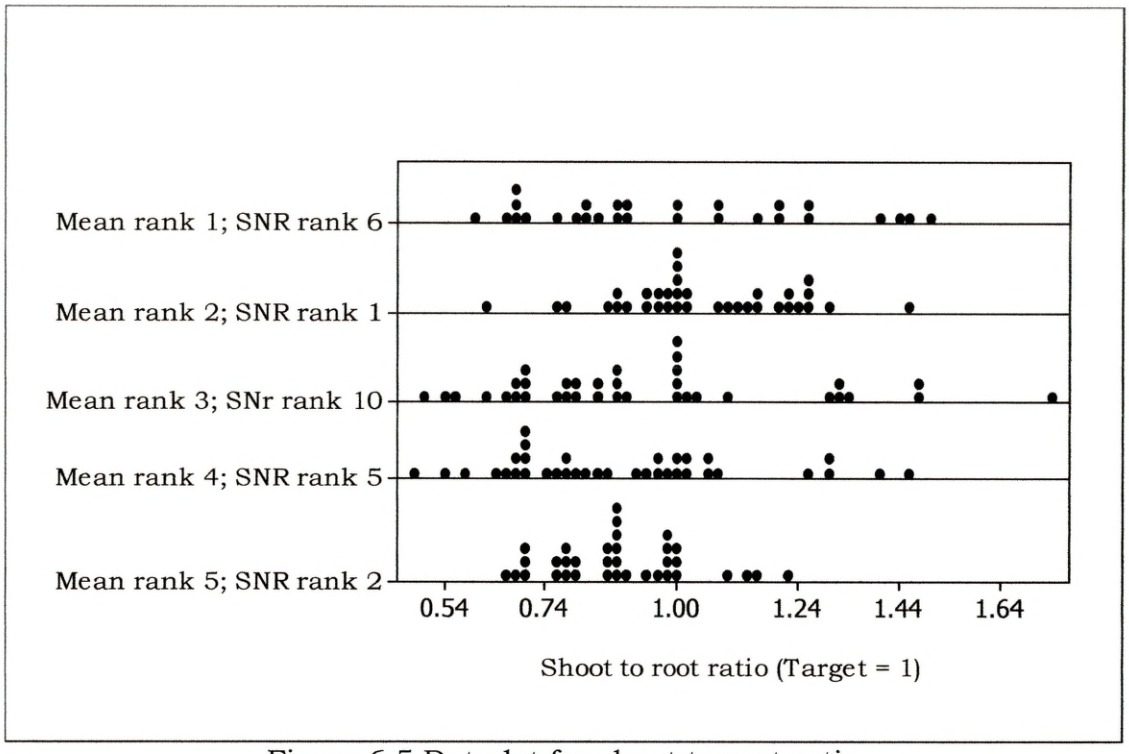


Figure 6.5 Dot plot for shoot to root ratio

6.4.5.1.3 Dry weight

ANOVA showed that no main effects or two-way interactions are significant for the dry weight of the seedlings. There was a significant positive Spearman's rank correlation (r_s = 0.780, $p<0.05$) between the mean and SNR values. The best five treatments for largest dry weight using mean and their corresponding SNR_{Large} are listed in Table 6.4.

Table 6.4. Five best treatments selected for largest dry weight (g) using the mean, their corresponding SNR_{Large} and rank in the full experiment

Drying method	**Drying time**	**Cooling rate**	**Warming rate**	**Media**	**Mean (g)**	**Rank Mean**	**SNR**	**Rank SNR**
Air	8	Rapid	Rapid	Soil	0.07831	1	-22.280	1
S.gel	4	Rapid	Slow	MS	0.02089	2	-37.490	7
S.gel	4	Rapid	Slow	Soil	0.01789	3	-40.504	22
S.gel	8	Slow	Slow	MS	0.01634	4	-37.561	9
S.gel	4	Slow	rapid	Soil	0.01632	5	-39.327	18

Figure 6.6 shows values for treatment combination with three highest mean and SNR values. Figure 6.6a shows the optimal treatment combination

identified by both ANOVA and SNR_{Large}. Figures 6.6b and c show the dot plot for the treatment combination with the second and third highest mean. It can be seen that the mean is affected by outliers and most values are relatively small. In contrast, Figures 6.6d and e each shows a constant sets of values which have the second and third highest SNR_{Large}. This again, demonstrates the robustness of the treatment combination with large SNR.

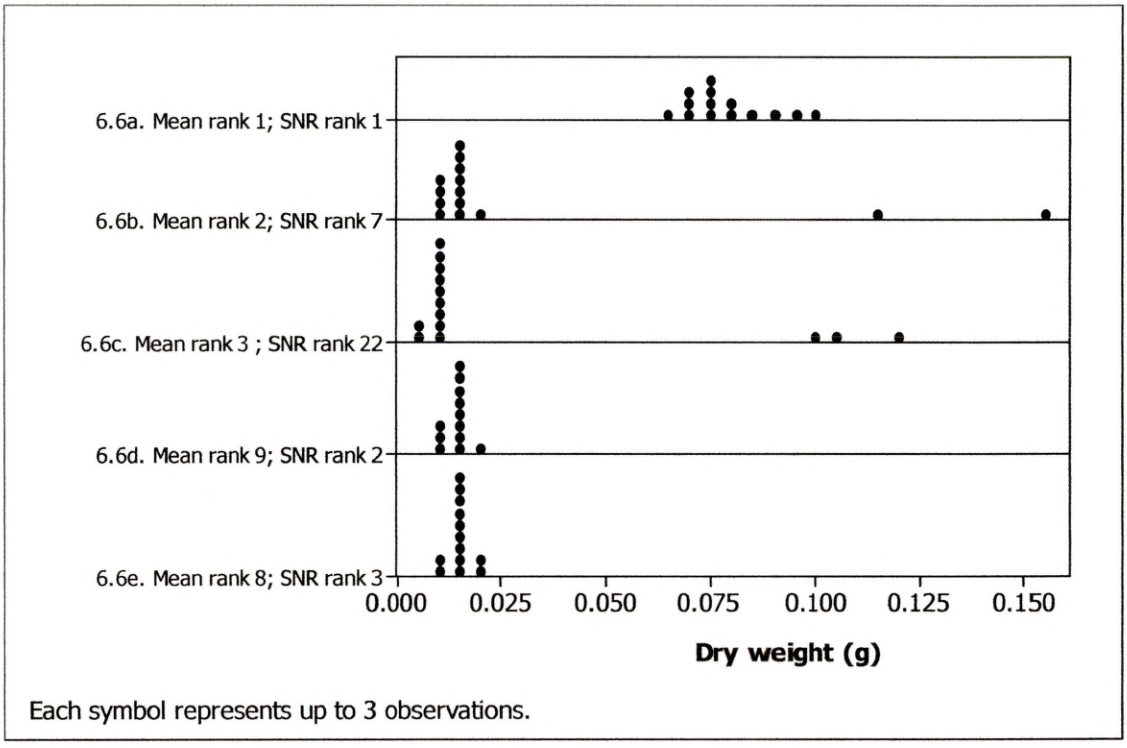


Figure 6.6 Dot plot for dry weight

6.4.5.2 Comparisons of half fractional replicates with full experiment for ANOVA and SNR

6.4.5.2.1 Sprouting day

The stepwise regression output shows that all main effects and two-way interactions are significant (Table 6.5). However, the model was selected at step three with R^2 value of 88.117%, since including other interactions in the model does not increase the R^2 value much. Though the main effects of drying method and cooling rate were not significant, their interaction had a significant effect on the sprouting day.

Table 6.5. The stepwise regression analysis output for sprouting day

Step	1	2	3
Constant	4.599	4.599	4.599
M	-0.779	-0.779	-0.779
T-Value	-5.680	-6.910	-8.390
P-Value	0.000	0.000	0.000
C*DM		0.313	0.313
T-Value		2.770	3.370
P-Value		0.016	0.006
DT			-0.249
T-Value			-2.680
P-Value			0.020
R-Sq	69.77	81.01	88.11

Therefore, the regression model for this response variable is:
Sprouting day = 4.599 -0.779 M -0.313C*DM – 0.249 DT.

The best treatment combination is when drying time at -1 level (4h), media -1 (soil), cooling rate –1 (slow) and drying method -1 (air) or cooling rate +1 (rapid) and drying method +1 (silica gel). The above regression equation was used to calculate the fitted values for this response variable for the full experiment. ANOVA including all main effects and two-way interactions showed that drying time [t(26)=4.24, p=0.000] and medium [t(26)=11.16, p=0.000] have a significant effect on sprouting day. A significant two-way interaction was noted for the drying method with cooling

rate [t(26)=-2.80, p=0.010] which is in agreement with full factorial experiment.

The same method was used for Taguchi analysis but using SNR as variable. As with the fractional factorial experiment, the Taguchi L_{16} experiment also showed a significant effect of drying time (t(26)=-4.17, p=0.000) and medium (t31)= -10.82, p=0.000) on sprouting day. There was also a significant two-way interaction between drying method and cooling rate (t(26)=2.54, p=0.017) for this analysis. Table 6.5 shows the five best treatments selected for the smallest sprouting day using the mean, their corresponding SNR_{Small} and rank.

Table 6.6. Five best treatments selected for the smallest mean sprouting day their corresponding SNR_{Small} and rank compared to the fitted values in the half replicated experiment

DM	CR	WR	Observed Mean (Rank)	Fitted Mean (Rank)	Observed SNR (Rank)	Fitted SNR (Rank)
Air	Slow	Rapid	2.97 (1)	3.34 (3.5)	-9.74 (1)	-10.75 (4.5)
Air	Rapid	Rapid	3.07 (2)	3.6 (5.5)	-9.91 (2)	-11.16 (6.5)
S.G	Rapid	Slow	3.29 (3)	3.3 (1.5)	-10.46 (3)	-10.67 (2.5)
S.G	Slow	Rapid	3.42 (4)	3.8 (7.5)	-10.83 (4)	-11.62 (8.5)
Air	Rapid	Slow	3.43 (5)	3.6 (5.5)	-10.90 (5)	-11.16 (6.5)

Note: For all the treatments selected above, the seeds were dried for 4 h and germinated in soil substrate. The values in the brackets show the rank order. DM=drying method, CR=cooling rate and WR = warming rate.

The Spearman's rank analysis showed a significant positive correlation between the ranks of observed and fitted means (r_s = 0.916, p<0.05) and also between the ranks of observed and fitted SNR_{Small} (r_s = 0.913, p<0.05) indicating that same conclusions are obtained using full and fractionally replicated experiment for both factorial and Taguchi methods.

6.4.5.2.2 Shoot to root ratio

The regression model from the stepwise regression analysis for this response variable is:

- Mean shoot to root ratio = 0.740 –0.277 media –0.0001 cooling rate +0.023 drying method -0.065 drying method*cooling rate.

- $SRN_{Nominal}$ = 8.20 –2.95 media –0.580 cooling rate + 0.529 cooling rate*media.

Table 6.7. Five best treatments selected for the smallest shoot to root ratio closer to one their corresponding $SNR_{Nominal}$ and rank compared to the fitted values in the half replicated experiment

	DM	DT	CR	WR	Observed Mean/$SNR_{Nominal}$ (Rank)	Fitted Mean/ $SNR_{Nominal}$ (Rank)
1	S.G	4	Rapid	Rapid	0.98 (1)	0.97 (2.5)
1	S.G	4	Slow	Rapid	15.06 (1)	11.99 (7.5)
2	S.G	4	Slow	Rapid	1.04 (2)	1.10 (14.5)
2	Air	4	Slow	Rapid	14.86 (2)	12.52 (3.5)
3	S.G	8	Rapid	Slow	0.93 (3)	0.97 (2.5)
3	Air	8	Slow	Slow	12.54 (3)	12.52 (3.5)
4	Air	8	Rapid	Slow	0.89 (4)	1.06 (6.5)
4	Air	4	Slow	Slow	12.75 (4)	12.52 (3.5)
5	Air	4	Slow	Rapid	0.88 (5)	0.93 (10.5)
5	Air	8	Rapid	Slow	11.62 (5)	9.48 (15.5)

Note: For all the treatments selected above, the seeds were germinated in soil substrate. The values in the brackets show the rank order. DM=drying method, DT=drying time, CR=cooling rate and WR = warming rate. The rows in brown shows the treatment combinations selected for $SNR_{Nominal}$ values.

There was a significant positive Spearman's rank correlation between the ranks of observed and fitted means (r_s = 0.885, $p<0.05$) and also between the ranks of observed and fitted SNR_{Small} (r_s = 0.882, $p<0.05$) indicating that almost similar conclusions are obtained using full and fractionally replicated experiment for both factorial and Taguchi methods. The summary of this analysis is shown in Table 6.8. The same method as above was used to compare conclusions from full and half fractionally replicated experiment for dry weight response variable and the results are summarised in Table 6.8.

For quarterly replicated experiments for both factorial and Taguchi methods, again the same procedure as described above was used to compare their conclusions with the full experiments. These results are also summarised in Table 6.8. These showed that quarterly replicated experiments also produced similar conclusions as the full and half fractional experiments except the two-way interactions could not be investigated in these quarter experiments.

6.4.6 Discussion

C. siamea seeds were successfully cryopreserved for the first time using desiccation protocol with survival above 70% after cryopreservation. This was facilitated by application of efficient experimental designs using full, fractional factorial and Taguchi methodologies.

6.4.6.1 Experimental designs and analysis

Six different experimental designs were compared for the cryopreservation *of C. siamea* seeds. The full experiment had 32 treatment combinations and was analysed using both ANOVA and SNR methodologies. The treatment combinations were ranked for smallest sprouting day, shoot to root ratio closest to one and largest dry weight. The relevant fractional factorial and Taguchi experiments were designed and the data were also analysed using both ANOVA and SNR. For these fractional replicates, though the same numbers of runs were conducted, different treatment combinations were selected using factorial and Taguchi methods. As described previously in Section 4.2.2, Taguchi uses orthogonal arrays and associated linear graphs to allocate factors to the columns to design an experiment. As a consequence, the treatment combinations selected were not the same as for the one selected in the factorial fractional experiments, as shown in Table 6.1.

For the half replicated factorial experiment, resolution V with design generator I=ABCDE was used. This design allows the investigation of all main effects and two-way interactions. However, in the quarterly replicated experiment, the highest resolution available is resolution III with design generator I=ABC. In this design, all main effects are aliased with two-way interactions. It is therefore, not possible to investigate the two-way interactions in this design. This was also the case for Taguchi designs, were it was possible to investigate all main effects and two-way interactions in half replicated experiment but in quarterly replicated experiment only main effects were investigated.

For sprouting day (smaller is better), the significant main effects were identical for the full, half and quarterly replicated experiments using either ANOVA or SNR (Table 6.8). Though interaction terms were not investigated in the ¼ replicate, the significant main effects were the same as in the larger experiments. The rank order of treatment combinations for the mean and SNR was highly significantly correlated for all experiments using either ANOVA or SNR.

For shoot to root ratio (nominal is best), only the soil main effect was significant for of the full, half and quarterly replicated experiments using ANOVA (Table 6.8). This effect was also significant using SNR which showed that cooling rate was significant for all experiments. Similarly, the SNR identified significant interactions not found using ANOVA. However, these significant interactions were not found in the half replicate. There was a highly significant rank correlation between treatment combinations for the mean and SNR (Table 6.8).

No significant effect was found in any experiment using either ANOVA or SNR for dry weight (larger is better), therefore, no comparison between ANOVA and SNR was carried out.

Some differences in determining the optimal treatment were found between ANOVA and SNR analyses of the same size experiment, especially in the shoot to root ratio and dry weight assessments (Table 6.8). For these cases, the Taguchi choice provided a more robust solution. The dot plots

showed that the treatments chosen using SNR are more consistent compared to those chosen using ANOVA (Figures 6.5. and 6.6).

This study shows that the full experiment was not necessary, since the fractional replicates chosen provided the same conclusions as the full experiments. This conclusion was consistent for analyses using either ANOVA or SNR. The half replicated experiment chosen allowed the investigation of all two-way interactions. However, it was not possible to investigate all two-way interactions in the quarterly replicated 2^5 experiments due to insufficient degrees of freedom. Therefore, care must be taken when the reduction in experimental size prevents the investigation of potentially important two-way interactions. In this case, the quarter fractional experiment is useful if only main effects and a limited number of interactions are of interest since only very simple models are available for this design.

Fractional factorial designs reduce the number of observations required (Babiak *et al.,* 2000), and such a reduction is especially suitable for screening or preliminary experiments, to determine those few experimental factors, out of many candidates, that have a significant effect on the results (Wu and Hamada, 2000). By using the fractional designs, the two-level approach can be extended to many factors. Therefore, these designs of experiments are ideal for screening many factors to identify the vital fewer that significantly affect the responses.

6.4.6.2 Post-cryopreservation recovery

A decreased deterioration in storage is important when the preserved species are in danger of extinction in the wild due to their low population numbers or restricted distribution. In many cases, desiccation-tolerant seeds can be immersed directly into liquid nitrogen without a decrease in germination after recovery providing the moisture content is kept within a reasonable range i.e. it is lower than freezing limit but not so low as to produce damage by fast cooling (Stanwood, 1985).

This study shows that drying method did not have a significant effect on any response variable for any experiment. It has been reported previously that rapid drying such as this resulted in viability loss for seeds with relatively high water content (Kioko *et al.*, 2000). However, for *C. siamea* seeds rapid drying does not affect their germination and high viability (above 80%) and vigour was maintained for both drying methods.

Desiccation time had a significant effect only on the sprouting day of the seeds for all experimental designs. It was noted that sprouting was more rapid after 4hr drying compared to 8hr dried seeds. This could be because 8hr dried seeds might have undergone desiccation stress and took longer to germinate which may explain why sprouting was delayed for these seeds. Walters *et al.*, (2001) showed that seed water status has a tremendous effect on seed germination and it could be manipulated to improve germination and stress conditions. As a general practice, it is recommended that seeds with low moisture content below 8-12% be humidified to avoid imbibition injury during germination (Bradford, 2002). Gonzalez-Benito *et al.*, (1998), showed that for *Centaurea hyssopifolia* seeds, which have a hard seed coat, humidification has not only reduced imbibition damage but also softened the seed coat and promoted germination.

Recovery after cryopreservation is largely dependent on reducing the impact of two injurious effect; dehydration and intracellular ice formation (Mazur, 2004). Both slow and rapid cooling were tested on the orthodox seeds. In slow cooling, ice formation is initiated outside the cell. Extra-cellular ice initiates cryo-dehydration caused by a vapour pressure deficit in relation to the cell's extra-and intra-cellular compartments. Unfrozen water moves from inside to outside of the cell in order to maintain the equilibrium, thereby increasing cell viscosity. Therefore, the cells will be vitrified and form a glassy state instead of ice crystal (Mishima and Stanley, 1998). This phenomenon can be cryo-protective as it reduces the amount of freezable water in the cells. However, if the dehydration effect is too great, cell damage occurs as a result of the reduced cellular water content. Ultra-rapid cooling can be applied if cells are sufficiently dehydrated and/or exposed to cryoprotectants so that they become vitrified or ice crystals formed were so small that they so not cause injury.

ANOVA shows that there is no difference between slow and rapid cooling for all three response variables. However, Taguchi analysis showed a significant effect of cooling rate for all designs showing that rapid cooling was the preferred method for *C. siamea* seeds cryopreservation. This is perhaps because the seeds have been desiccated to moisture contents of 14% and below at which all the freezable water has been removed. Therefore, ice-induced freezing injury does not occur resulting in a high survival rate after cryopreservation. Wesley-Smith *et al.*, (2001) reported that rapid cooling is best applied to tissues, which are highly cytoplasmic and contain very little water such as orthodox seeds and slow freezing is more suitable for seeds that have relatively high water content. However, this may be moderated in protocols that utilize chemical cryoprotectants.

Warming rate is as important to cell survival as the cooling rate, and will depend on what state of water has formed during cooling, e.g. ice or amorphous glass (Mazur, 2004). There is a possibility that large crystals grow from small crystals on rewarming. If glass has formed during cooling, and if the glass is less stable, during rapid warming, de-vitrification will occur and ice will again form. But if the glass is stable, a glass transition will take place and no ice will form. Rapid warming is recommended to prevent re-growth of ice crystals during thawing (Withers, 1985; Schrijnemakers and Van Iren, 1995; Van Iren *et al.*, 1995). This study shows that warming rate has a significant interaction with drying time for shoot to root ratio in Taguchi full experiment. Four hours drying time associated with rapid warming gave shoot to root ratio closer to one (Table 6.8). This may be due to there being less water in the seeds (< 14%) and the formation of a stable glassy state during cooling and the stabilization of a glass transition on re-warming.

Recovery medium had a significant effect on sprouting day and shoot to root ratio. Seeds sown in soil sprouted rapidly compared to those in MS medium. Though the shoot to root ratio can be influenced by many factors (Paz, 2003), it was found that for seeds sown in the soil substrate, had a ratio nearer to one. A smaller shoot to root ratio was observed on MS medium. ANOVA based analyses revealed no difference between slow and

rapid cooling or warming, whereas, the Taguchi approach demonstrated that rapid cooling and warming generally gave a shoot to root ratio closer to one.

6.4.7 Summary of findings

1. *Cassia siamea* species was successfully cryopreserved for the first time using either desiccation protocol with high survival (>70%) after cryopreservation.

2. Taguchi experimental design using SNR was successfully applied to the cryopreservation experiments providing similar results compared to traditional factorial designs with almost the same conclusions for both factorial and Taguchi methods (Table 6.8).

3. The analyses concluded that fractional replicates of full factorial experiments are sufficient for screening experiments since these two experiments provided the same conclusions. This is also true for Taguchi designs.

6.4.8 Progress towards the confirmatory experiment

The results of this experiment showed that the factors that affect most of the response variables were desiccation time, warming rate and recovery media. Hence, A confirmatory experiment was carried out on this species with these three factors at three levels each in the next section (Section 6.5). For drying time, however, four hours desiccation gave the more rapid germination rate, seeds dried for eight hours gave higher germination.

Table 6.8. Summary of key findings for *C. speciosa* cryopreservation experiment with five factors at two levels

Experimental design → / Response variables ↓	Full factorial (2^5)	Factorial ½ fractional (2^{5-1})	Factorial ¼ fractional (2^{5-2})	Taguchi L_{32}	Taguchi L_{16}	Taguchi L_8
Sprouting day						
Significant main effect						
Drying time	S,p=0.001 (4 h)	S,p=0.000, (4 h)	S,p=0.000,(4 h)	S,p=0.001(4 h)	S,p=0.000, (4 h)	S,p=0.000,(4 h)
Media	S,p=0.000,(soil)	S,p=0.000, (soil)	S,p=0.000,(soil)	S,p=0.000,(soil)	S,p=0.000,(soil)	S,p=0.000,(soil)
Significant interaction	S,p=0.019	S,p=0.010	-	S,p=0.029	S,p=0.017	-
Drying method*cooling rate	1.000(p<0.05)	-	-	1.000 (p<0.05)	-	-
Spearman's rank correlation coefficient, r_s,	-	0.916(p<0.05)	0.885(p<0.05)	-	0.848(p<0.05)	0.746(p<0.05)
Observed mean versus observed SNR	-	0.913(p<0.05)	0.882(p<0.05)	-	0.738(p<0.05)	0.663(p<0.05)
Observed mean versus fitted mean	-	1.000(p<0.05)	1.000(p<0.05)	-	1.000(p<0.05)	0.924(p<0.05)
Observed SNR versus fitted SNR						
Fitted mean versus fitted SNR						
Shoot to root ratio						
Significant main effect						
Cooling rate	NS	NS	NS	S,p=0.010,(rapid)	S,p=0.000(rapid)	S,p=0.000(rapid)
Media	S,p=0.000](soil)	S,p=0.000,(soil)	S,p=0.000,(soil)	S,p=0.000,(soil)	S,p=0.000,(soil)	S,p=0.000(soil)
Significant interaction						
Drying method*cooling rate	S,p=0.008	S,p=0.002	-	NS	S, p=0.028	-
Media*cooling	NS	NS	-	S, p=0.016	NS	-
Drying time*media	NS	NS	-	S, p=0.029	NS	-
Drying time*warming rate	NS	NS	-	S, p=0.009	NS	-
Spearman's rank correlation coefficient, r_s,	0.950(p<0.05)	-	-	0.950(p<0.05)	-	-
Observed mean versus observed SNR	-	0.820(p<0.05)	0.796(p<0.05)	-	0.854(p<0.05)	0.878(p<0.05)
Observed mean versus fitted mean	-	0.721(p<0.05)	0.713(p<0.05)	-	0.876(p<0.05)	0.795(p<0.05)
Observed SNR versus fitted SNR	-	0.643(p<0.05)	0.762(p<0.05)	-	0.695(p<0.05)	0.781(p<0.05)
Fitted mean versus fitted SNR						
Dry weight						
No significant main effect or interaction noted						

NS= Not significant, S= Significant, parentheses show levels associated with better treatments

6.5 comparing Full 3^3 Factorial Experiment With Fractional Replication and Taguchi Techniques For the Cryopreservation of Cassia siamea Seeds

6.5.1 Introduction

In the previous section, a cryopreservation experiment which comprises five factors at two levels each was carried out on *C. siamea* seeds (Section 6.4). However, the results showed that only three out of five factors had a significant effect on the response variables. Based on this information, a confirmatory experiment with three factors at three levels was designed. The factors that affect most of the response variables in the previous experiment were desiccation time, warming rate and recovery media. For drying time however, though four hours desiccation gave the more rapid germination rate, seeds dried for eight hours gave higher germination (Table 6.1). Therefore, seven and nine hour drying time, in addition to eight hours were investigated to test for optimization. For the warming rate, besides rapid and slow warming, a third level (slow-rapid) was also assessed. Two-step slow-rapid warming maybe important in glass stabilisation and until now has not been applied for cryopreservation experiments of tropical forest species at FRIM. The information gained from this experiment may be very useful and could help understand the behaviour of intermediate and recalcitrant seeds. Soil substrate was included since the seeds will be germinated in actual field conditions (ISTA, 1999). The factors and their levels for the confirmatory experiment are as follow:

A: Drying time (level -1=7h, 0=8h and 1=9h)
B: Warming rate (level -1=rapid, 0=slow-rapid, 1=slow)
C: Recovery media (level -1=filter paper, 0=soil and 1=MS medium)

6.5.2 Materials and methods

The methodology of this experiment is similar to the first experiment. The seeds were air desiccated for seven, eight or nine hours in a laminar flow cabinet followed by rapid cooling by direct immersion in liquid nitrogen. For rapid warming, the seeds were held for four minutes in a water bath at 40°C

immediately after removal from liquid nitrogen (LN). For slow-rapid warming, the seeds were taken out from LN and held at ambient temperature for two minutes and then transferred to 40°C in the water bath for another two minutes. For slow warming, the seeds were kept at room temperature for four minutes. Therefore, the seeds were immersed in the water bath for four, two and zero minutes for rapid, slow-rapid and slow warming respectively. The re-warmed seeds were germinated either on filter paper, or soil and on MS medium. Assessments of germination day, shoot to root ratio and dry weight were carried out as described in the first experiment.

The main objectives of this experiment are to examine the robustness of results obtained in the first experiment to obtain more consistent conclusions. This is attained by firstly reducing the number of factors from five to the three significant factors, secondly, three levels are used for each factor to allow a quadratic relationship to be investigated for the quantitative factors and another medium to be investigated.

6.5.3 Design of experiment and data analysis

The experiment was designed as a general full factorial and Taguchi experiments with three factors at three levels each. To allow comparisons, the full factorial and one third fractional factorial were analysed using ANOVA whilst, the Taguchi full (L_{27}) and one third fractional (L_9) experiments used stepwise regression on the SNRs. Forty replicates (one seed is a replicate) were used per treatment combination. Table 6.9 summarises the treatment combinations selected for each experimental design.

Covariance analysis was used since the levels of two of the factors (desiccation time (7, 8 and 9hr) and warming rate (time in the water bath 4min (rapid), 2min (slow-rapid) and 0min (slow)) were evenly spaced. Therefore, in this experiment, the data were analysed with desiccation time and warming rate as covariates and recovery medium was used as the model with filter paper as the base level.

Table 6.9. Treatment combination selected for each experimental design and the germination of cryopreserved *C. siamea* seeds

Run	DT	WR	M	$3^3 / L_{27}$	3^{3-1}	L_9	Germination(%)
1	7h	Rapid	Filter paper	Y	Y	Y	77.5
2	7h	Rapid	Soil	Y			77.5
3	7h	Rapid	MS	Y			85.0
4	7h	Slow-rapid	Filter paper	Y			77.5
5	7h	Slow-rapid	Soil	Y	Y	Y	80.0
6	7h	Slow-rapid	MS	Y			77.5
7	7h	Slow	Filter paper	Y			82.5
8	7h	Slow	Soil	Y			82.5
9	7h	Slow	MS	Y	Y	Y	77.5
10	8hr	Rapid	Filter paper	Y	Y	Y	82.5
11	8hr	Rapid	Soil	Y			85.0
12	8hr	Rapid	MS	Y			82.5
13	8hr	Slow-rapid	Filter paper	Y			80.0
14	8hr	Slow-rapid	Soil	Y	Y	Y	82.5
15	8hr	Slow-rapid	MS	Y			77.5
16	8hr	Slow	Filter paper	Y			87.5
17	8hr	Slow	Soil	Y			82.5
18	8hr	Slow	MS	Y	Y	Y	77.5
19	9hr	Rapid	Filter paper	Y	Y	Y	85.0
20	9hr	Rapid	Soil	Y			80.0
21	9hr	Rapid	MS	Y			80.0
22	9hr	Slow-rapid	Filter paper	Y			90.0
23	9hr	Slow-rapid	Soil	Y	Y	Y	80.0
24	9hr	Slow-rapid	MS	Y			85.0
25	9hr	Slow	Filter paper	Y			80.0
26	9hr	Slow	Soil	Y			80.0
27	9hr	Slow	MS	Y	Y	Y	77.5

DT=drying time, WR=warming rate, M=medium, MS=Murashige and Skoog (1962), Y=selected treatments

6.5.4 Results

6.5.4.1 Comparisons of ANCOVA and SNR for the full experiments

6.5.4.1.1 Sprouting day

ANCOVA showed that drying time had significant linear effect (Figure 6.7) on sprouting day [F(1,872) = 6.22, p=0.013] but warming rate does not [F(1,872) = 0.02, p=0.880].

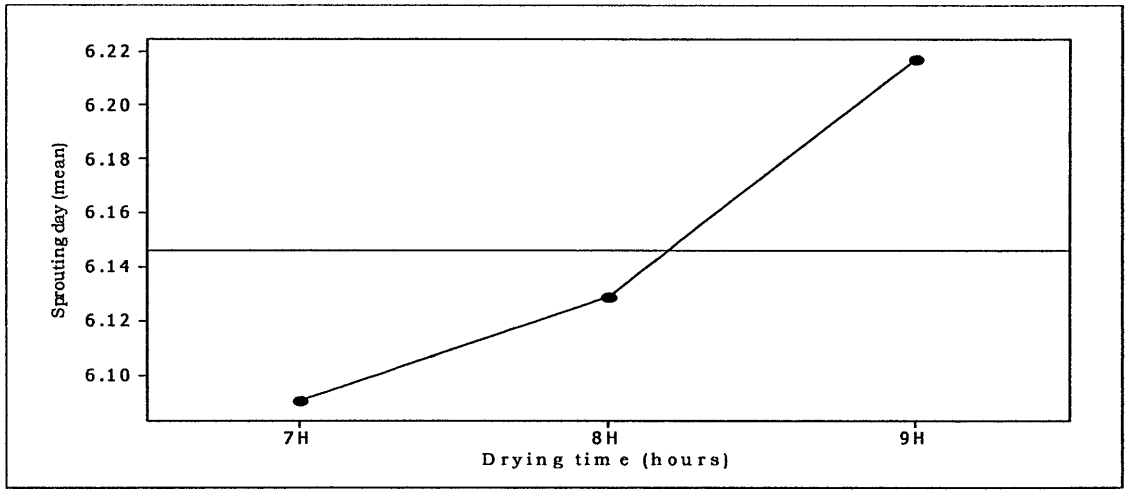


Figure 6.7 Main effect plot of drying time showing linear effect on sprouting day

Both recovery media, MS [F(1,872) = 487.58, p=0.000] and soil [F(1,872) = 2202.76, p=0.000] have significant effects on sprouting day compared to filter paper, provided other conditions are fixed. MS media and soil delayed sprouting by 1.44 and 3.04 days respectively compared to filter paper (Figure 6.8). There were also a number of significant two-way interactions as listed in Table 6.13.

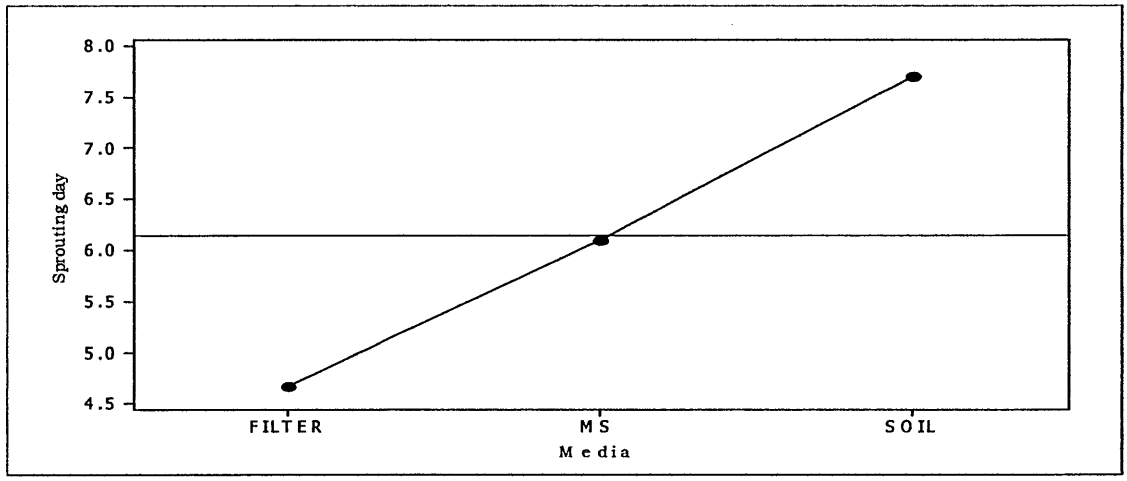


Figure 6.8 Main effect plot for media showing filter paper as the optimal with quickest sprouting day

The Taguchi analysis showed that drying time and warming rate do not have a significant effect on sprouting day. Both recovery media, MS [F(1,22)=97.65, p=0.000] and soil [F(1,22)=342.35, p=0.000] have significant and therefore different effects on sprouting day as compared to filter paper, provided other conditions are fixed. MS media decreased SNR values for sprouting day by 2.30 compared to filter paper. MS media delayed sprouting day by an average of 1.4 days compared to filter paper. Soil decreased SNR values for sprouting day by 4.30 compared to filter paper. Soil medium delayed sprouting day by an average of three days compared to filter paper. These results are in agreement with factorial experiment. However, no significant two-way interaction was noted in this analysis.

The Spearman's rank correlation between the observed mean and SNR ranks was one, showing that both methodologies have exactly the same rank of treatment combinations. Table 6.10 shows the treatment combinations in the first five rank selected for smallest sprouting day using the mean, their corresponding SNR_{Small} and ranks.

Table 6.10. Treatment combinations in first five rank selected for smallest sprouting day using the mean, their corresponding SNR_{Small} and ranks

Drying time	Warming rate	Mean (day)	Rank Mean	SNR	Rank SNR
8	Slow-rapid	4.4688	1	-13.0843	1
8	Slow	4.5714	2	-13.3099	2
9	Slow	4.5938	3	-13.3307	3
7	Slow	4.6061	4	-13.3397	4
7	Slow	4.6061	5	-13.3640	5

Note: For all the treatments selected above, the seeds were germinated in filter paper medium

6.5.4.1.2 Shoot to root ratio

ANCOVA showed that drying time has a significant linear effect (Figure 6.9) on shoot to root ratio [F(1,869)=18.07, p=0.000]. Figure 6.9 shows that nine hours desiccation time gives shoot to root ratios closer to one, which is preferred for this response variable. Warming rate does not have a significant effect on shoot to root ratio.

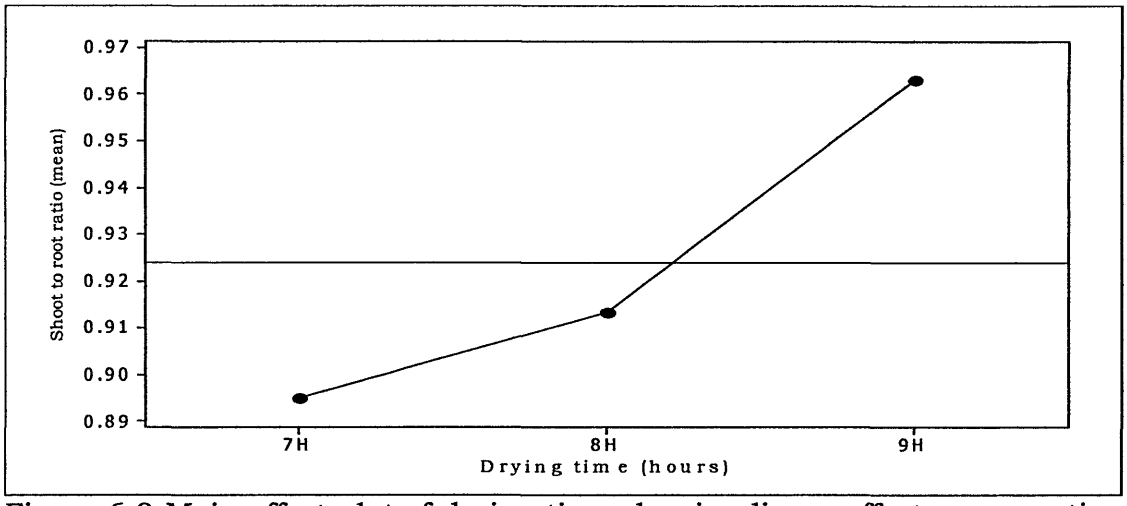


Figure 6.9 Main effect plot of drying time showing linear effect on sprouting day

Both recovery media, MS [F(1,117.511) = 568.23, p=0.000] and soil [F(1,79.878) = 1808.41, p=0.000] have significant effects on shoot to root ratio. Therefore, they have different effects on shoot to root ratio compared to filter paper provided other conditions are fixed. MS media decreased shoot to root ratio by an average of 0.42 compared to filter paper. Soil increased the shoot to root ratio by 0.74 compared to filter paper (Figure 6.8). Overall, it was noted that seeds grown on filter paper have shoot to root ratio closest to one (Table 6.8). Significant two-way interactions were observed between drying time and warming rate (p=0.011) and between drying time and MS medium (p=0.003).

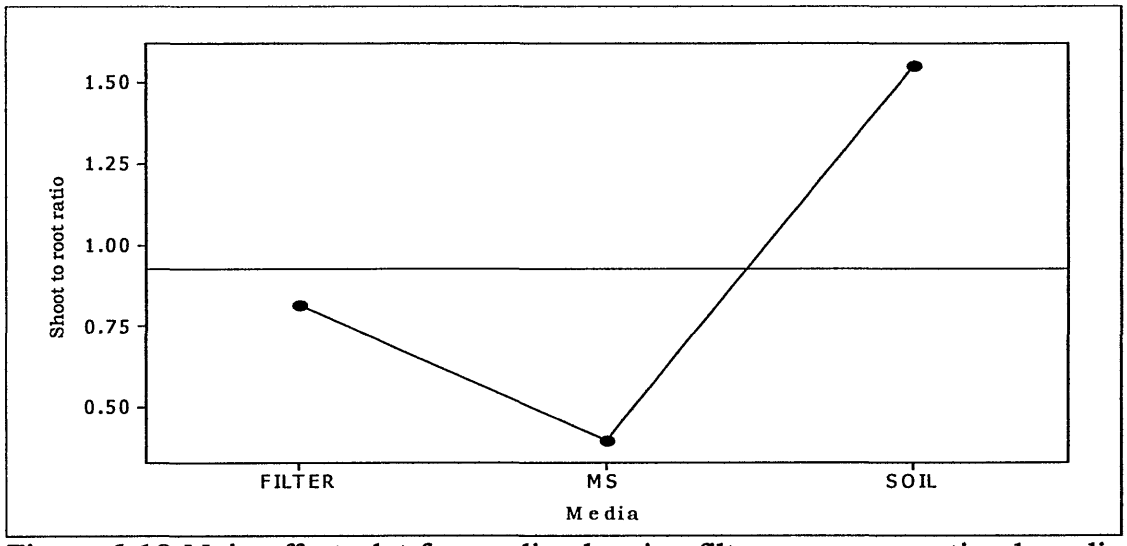


Figure 6.10 Main effect plot for media showing filter paper as optimal media for shoot to root ratio

Taguchi analysis showed that drying time and warming rate do not have a significant effect on shoot to root ratio SNR values. Both media, MS [F(1,22) = 67.24, p=0.000] and soil [F(1,22) = 68.41, p=0.000] have significantly different effects on sprouting day compared to filter paper provided other conditions are fixed. MS medium decreased SNR for shoot to root ratio by 7.4003 (decreased shoot to root ratio by 0.4) compared to filter paper. Soil decreased SNR for shoot to root ratio by 7.4646 (increased shoot to root ratio by 0.7) compared to filter paper. No significant two-way interaction was noted. The Spearman's rank correlation between the observed mean and SNR was one ($p<0.05$), showing that both methodologies have exactly the same rank of best treatment combinations. Table 6.11 shows the five best treatment combinations selected for shoot to root ratio closer to one using the mean, their corresponding $SNR_{Nominal}$ and ranks.

Table 6.11. Five treatment combinations with mean shoot to root ratio closest to one using the mean, their corresponding $SNR_{Nominal}$ and its rank

Drying time	Warming rate	Mean	Rank Mean	SNR	Rank SNR
7	Rapid	1.0003	1	16.3192	1
9	Slow	1.0131	2	13.3092	2
9	Slow-rapid	0.8647	3	13.2983	3
9	Rapid	0.8527	4	13.0596	4
7	Slow	0.8191	5	12.4739	5

Note: For all the treatments selected above, the seeds were germinated in filter paper medium

6.5.4.1.3 Dry weight

Both ANCOVA and Taguchi showed that there is no significant effect of any main effects or two-way interactions. Spearman's rank correlation coefficient between the observed means and SNR was 0.955 ($p<0.05$) showing that the rank orders for the treatment combinations for both methodologies are in general agreement. Table 6.12 shows the five best treatment combinations selected for largest dry weight using the mean, their corresponding SNR_{Large} and ranks.

Table 6.12. Five best treatment combinations selected for largest dry weight using the mean, their corresponding SNR_{Large} and ranks

Drying time	Warming rate	Media	Mean	Rank Mean	SNR	Rank SNR
8	Slow-rapid	Soil	0.014714	1	-36.9201	1
7	Slow-rapid	MS	0.014480	2	-37.4321	5
8	Rapid	Soil	0.014358	3	-37.0365	2
7	Slow	MS	0.014229	4	-37.5533	7
9	Rapid	Filter	0.014115	5	-37.5979	8

The dot plots for mean rank 1, 2 and 3 are shown in Figure 6.11. Figure 6.11(a) has the greatest mean (0.01471g) and largest SNR and therefore, in the first rank for mean and SNR. Figure 6.11(b) has the second greatest mean (0.01458g) but the data have three low outliers and is ranked fifth for SNR. Figure 6.11(c) has the third greatest mean (0.01435g), and the data are closely distributed (except for 3 outliers) compared to (b) and was in ranked second for SNR.

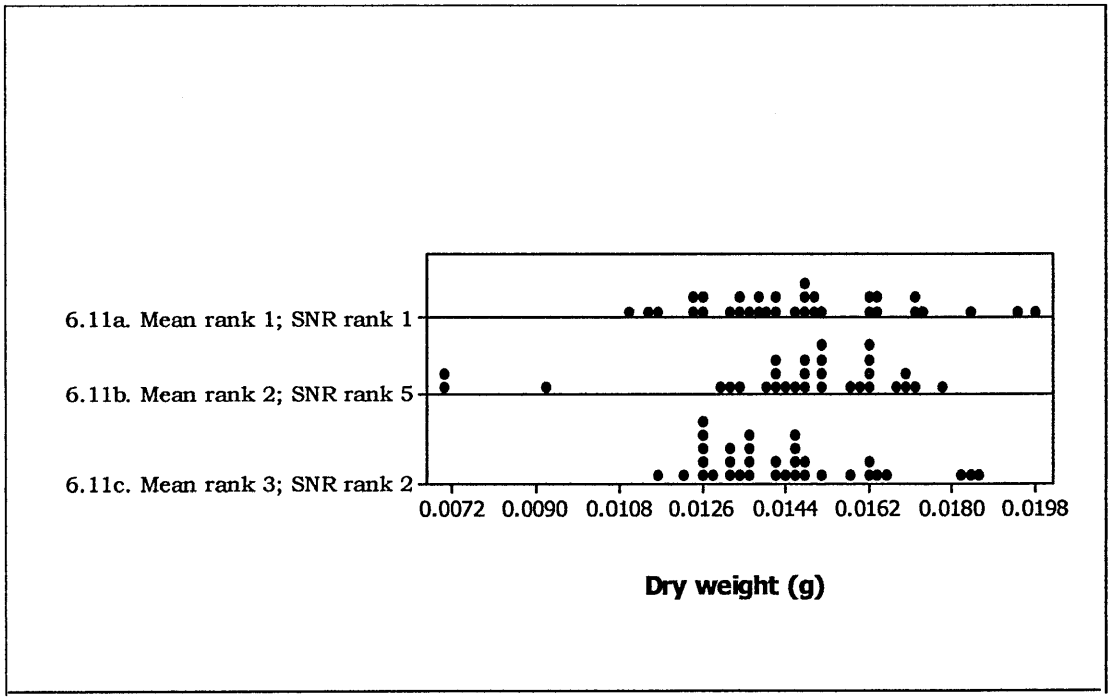


Figure 6.11 Treatment combination in rank one for mean and SNR (a);treatment with second greatest mean but in rank 5 for SNR (b); treatment with third greatest mean and in rank two for SNR (c).

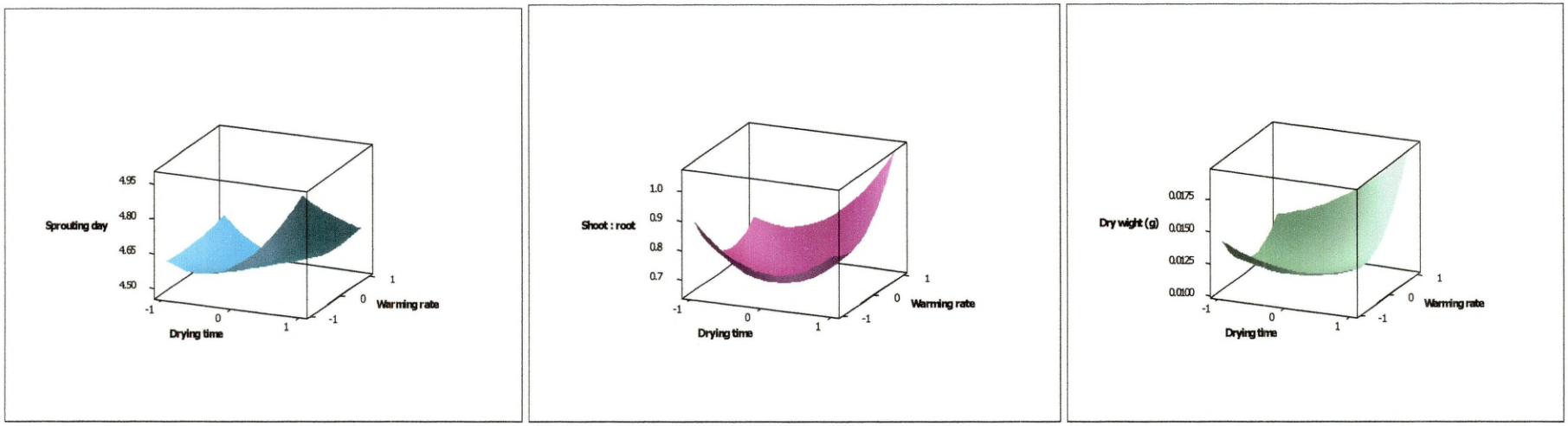
6.5.4.2 Comparisons of ANCOVA and SNR for the fractional experiments

The comparisons of fractional replicates are summarized in Table 6.13. Drying time was significant for sprouting day and shoot to root ratio response variables in full factorial experiment but not in any other experimental designs (Table 6.13). No two-way interactions could be investigated in either factorial (3^{3-1}) or Taguchi L_9 experimental designs due to insufficient degrees of freedom. Both Taguchi L_{27} and L_9 designs showed exactly the same significant effects. However, this was slightly different for full and one-third factorial designs as summarized in Table 6.13.

6.5.4.3 Response surface analysis

Since there was a highly significant effect of recovery media for sprouting day and shoot to root ratio, the experiment was split into three by media and analysed separately using response surfaces. Table 6.14 gives the summary of the results. Drying time had a significant effect on sprouting day in MS medium and soil. The response surface plots for all three response variables in all three germination media are shown in Figure 6.12. Warming rate had a quadratic effect on sprouting day in both MS medium and soil substrate (Figures 6.12b and c).

A significant quadratic effect for warming rate was also noted for shoot to root ratio in all germination media (Table 6.14). However, a significant quadratic effect of drying time was only noted for shoot to root ratio in soil and filter paper substrates (Table 6.14 and Figure 6.12). Warming rate showed a significant effect on dry weight of the seedlings in MS medium and filter paper substrate. The two-way interaction between drying time and warming rate was also significant for dry weight in all three media.

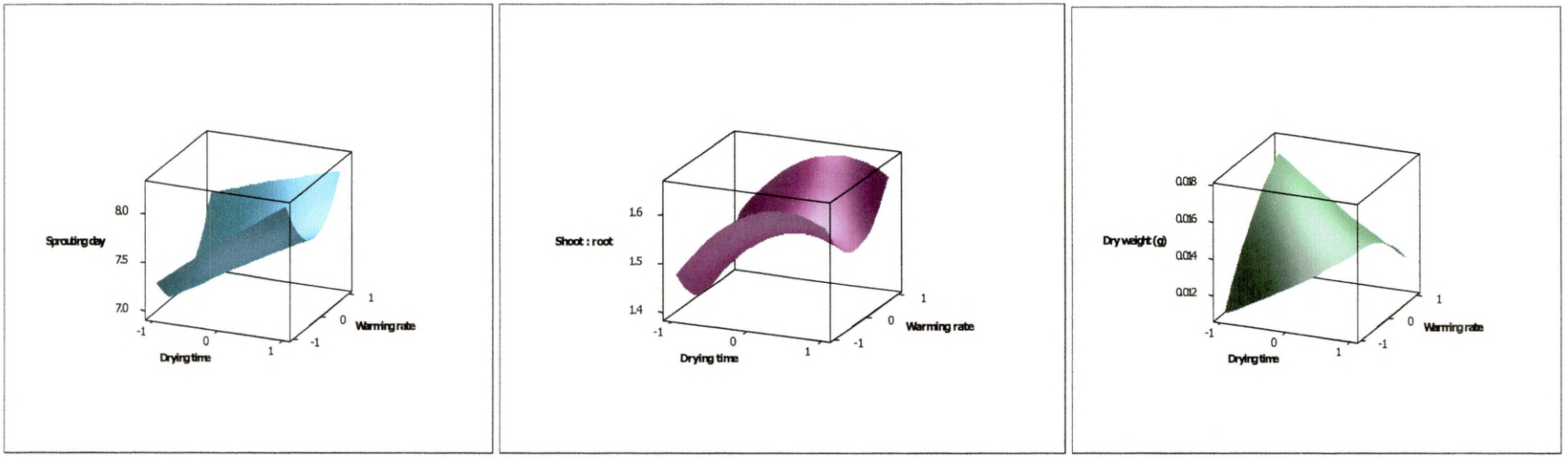


WR^2: Not significant
DT^2 : Not significant

WR^2: Significant (p=0.000)
DT^2 : Significant (p=0.000)

WR^2: Significant (p=0.047)
DT^2 : Not significant

Figure 6.12a Response surface plots for sprouting day, shoot to root ratio and dry weight for filter paper.
(Drying time –1=7h, 0=8h and 1=9h; warming rate –1= slow, 0=slow-rapid and 1=rapid; WR^2=quadratic effect of warming rate; DT^2= quadratic effect of drying time)

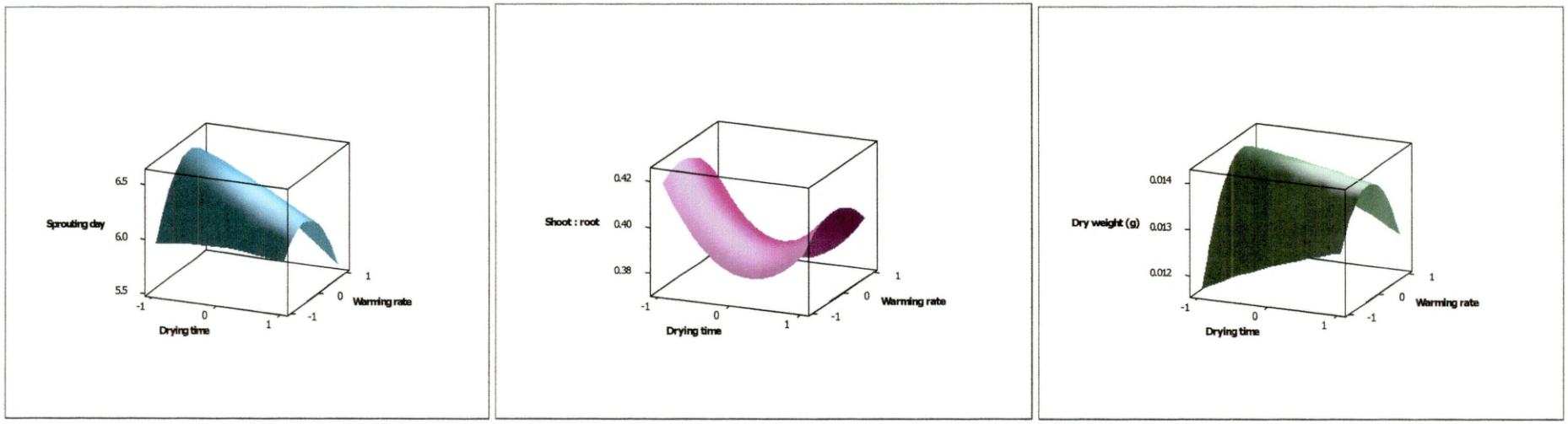


WR^2: Significant (p=0.000)
DT^2 : Not significant

WR^2: Significant (p=0.006)
DT^2 : Significant (p=0.028)

WR^2: Not significant
DT^2 : Not significant

Figure 6.12b Response surface plots for sprouting day, shoot to root ratio and dry weight for soil.
(Drying time –1=7h, 0=8h and 1=9h; warming rate –1= slow, 0=slow-rapid and 1=rapid; WR^2=quadratic effect of warming rate; DT^2= quadratic effect of drying time)



WR^2: Significant(p=0.000)
DT^2 : Not significant

WR^2: Not significant
DT^2 : Significant (p=0.008)

WR^2: Significant (p=0.000)
DT^2 : Not significant

Figure 6.12c Response surface plots for sprouting day, shoot to root ratio and dry weight for MS medium.
(Drying time –1=7h, 0=8h and 1=9h; warming rate –1= slow, 0=slow-rapid and 1=rapid; WR^2=quadratic effect of warming rate; DT^2= quadratic effect of drying time)

6.5.5 Discussion

6.5.5.1 Post-cryopreservation recovery

This is the first time cryopreservation has been applied to *C. siamea* seeds (also see Muthusamy *et al.*, 2005). Cryopreservation is a possible alternative for the long term-term storage for orthodox seeds (Salomao, 2002; Nunes *et al.*, 2003). An essential first step in orthodox seed cryopreservation is to determine the optimum (safe) moisture content, particularly for those with a high oil content. Salomao (2002), suggested that a moisture content between 3.8 and 11 percent is safe for the cryopreservation of most tropical orthodox tree seeds. The first experiment showed that germination of *C. siamea* seeds after cryopreservation was much higher after eight hours desiccation compared to four hours (Section 6.4, Table 6.1). In this experiment, the moisture content of the seeds declined from 20% to 10, 8.4 and 5.5% after seven, eight and nine hours respectively of air desiccation. ANCOVA showed that drying time had a significant linear effect on sprouting day and shoot to root ratio. As drying time increased, sprouting day was delayed and the seedlings had a larger shoot to root ratio (mainly due to shorter root development). This observation was also made in the previous experiment, where four hours desiccated seeds sprouted more rapidly compared to eight hours dried seeds.

Dehydration will exert stress causing a delay on sprouting day as a recovery response. Extreme desiccation did not have a harmful effect on the germination of oil-rich seed (Zhou and Bi, 1993). Some species such as rice, cucumber and soya bean were damaged by excessive dehydration (Zhi and Bi, 1991; Zhang *et al.*, 2004; Jing and Zheng, 1994). This is possibly because as seeds become too dehydrated, it is damaging to take up water too fast as imbibition injury could take place. Once a seed had dried, it is evident that water must be reabsorbed before metabolic activity can resume (Bradford, 2002). In some seeds, impermeable seed coats prevent water uptake, extending the quiescent period until weathering or biological action renders them permeable to water but slow the rate of imbibition to prevent damage that can occur if water is absorbed too rapidly (Jeller *et al.*, 2003). Other seeds hydrate very quickly when in contact with water. Thus, the initial rate of imbibition can vary widely depending upon the characteristic of

the testa and/or pericarp that surrounds the embryo (Bewley *et al.*, 2000). The initial phase of water uptake is a physical process driven by the water potential gradient between the seed and its environment (Hosnedl and Honsova, 2002). Following the initial phase of imbibition Phase I (Figure 6.13), seed water content usually reaches a plateau level and remains relatively constant or only slowly increases for a period known as the 'lag phase' or Phase II of imbibition (Figure 6.13). During this phase, metabolic processes are activated that are required for embryo growth and completion of germination. Phase III of imbibition is marked by an increase in seed water content due to absorption associated with the initiation of embryo growth (adapted from Bewley *et al.*, 2000).

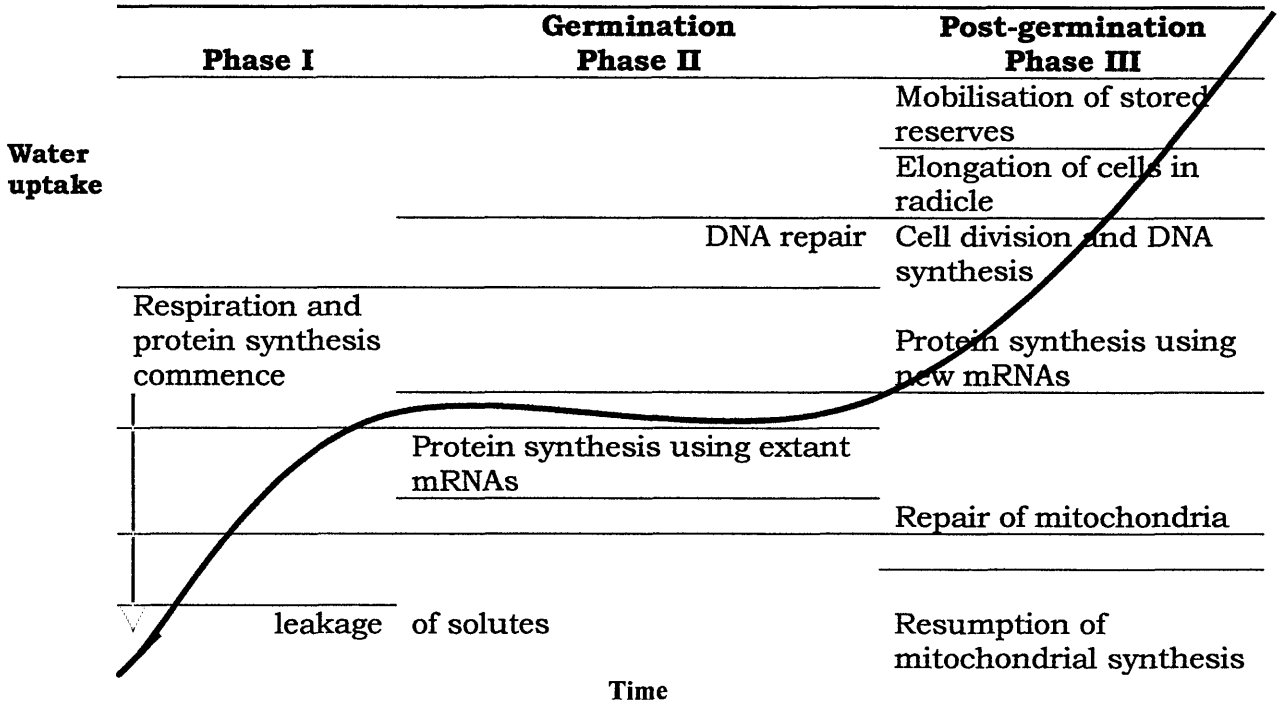


Figure 6.13 Triphasic pattern of water uptake during seed imbibition and associated metabolic events (Adapted from Bewley *et al.*, 2000)

One possible cause of problems with liquid nitrogen seed storage is the requirement to optimize both the rate of cooling and the subsequent warming and these must be determined empirically (Mazur, 2004). Rapid cooling, which is simple and does not require a programmable freezer was applied in this experiment since the previous experiment showed that cooling rate does not have a significant effect on this seed viability.

The rewarming of the cryopreserved seeds requires careful selection of heating rates sufficient to prevent significant thermal cracking, devitrification and recrystallization during heating. The use of carefully designed warming protocols is necessary to maximise seed viability and maintain structural integrity. In this experiment, three warming rates were tested. Though the previous experiment's results on this seeds showed that the warming rate had significant effect on most of the response variables, this experiment's results showed that all three levels of warming rate were not significant on any of the response variables. This could be due to the moisture content of the seed, which has been reduced below 10%. Therefore all the osmotically active water may have been removed and thus they are vitrified during cooling and formed stable glass transition during subsequent re-warming. It was reported that vitrified materials, which may contain appreciable thermal developed during cooling might require an initial brief slow warming followed by rapid warming steps (Brockbank *et al.*, 2003). The slow warming is reported to relieve residual thermal stresses and when this followed by rapid warming, it minimises the potential for devitrification and recrystalisation phenomena.

Recovery media had a significant effect on sprouting day and shoot to root ratio. MS medium and soil substrates delayed sprouting day compared to filter paper. Shoot to root ratio assessment also supports this finding. It was found that for seeds sown in soil substrate, the ratio is near to one, which is expected in normal seedling development. Smaller shoot to root ratios were observed on MS medium, since longer roots were developed to support the seedling development in a space limited environment.

6.5.5.2 Experimental designs and data analysis

The overall strategy for experiments one and two is that the first is a screening and the second is the confirmatory of the first. In the screening experiment there was an advantage to include many factors but with limited levels, in this case five factors at two levels each. According to Myers and Montgomery (1995), there are three choices to model a screening experiment such as linear, linear with centre point and Plackett-Burman designs.

Screening experiments isolate or identify those factors that are most important for the selected response variables. From these, conclusions and decisions may be made also to which factors are to be included in future experiments. This decision-making process involves both information from the statistical analysis and the researcher's own knowledge of the system, including other limiting factors or cost. Once the decision is made, the second step is the exclusion of those factors deemed to be of minor significance to the response variables (in this case drying method and cooling rate). Therefore, the second experiment was designed with only three factors. In this experiment there is the advantage of investigating an additional level for each selected factors. Response surfaces can be used to determine the changes in the response variables for any combination of changes in the factors (Myers and Montgomery, 1995). The response surface consists of a three-dimensional collection of all experimental points measured and plotted (as well as areas defined by these points) and is bounded by the limits of the actual experiment.

The advantages of a full factorial design include orthogonality, ability to exclude aliasing and the evaluation of all main effects and interactions (Box *et al.*, 1978). The disadvantages include time, cost and resource commitment. Fractional factorial designs can retain orthogonality while requiring fewer runs. However, doing fewer runs means acquiring less information. To ensure that subsequent run capture the most important information, the insignificant higher order or all interactions are usually ignored (Wu and Hamada, 2000).

In this experiment, the full factorial experiment showed significant effect of drying time for both sprouting day and shoot to root ratio response variables, however, fractional factorial design did not show a significant effect of drying time for both these response variables. Taguchi full and fractional replicates did not show significant effects of drying time for both sprouting day and shoot to root ratio response variables (Table 6.13). Both fractional factorial and Taguchi L_9 designs showed the same results. Overall, all three (Taguchi L_{27}, L_9 and fractional factorial 3^{3-1}) designs showed similar conclusions (Table 6.13). Some differences were found in determining the optimal treatment between ANCOVA and SNR analyses, which was also

noted in experiment one. Taguchi designs showed more robust conclusions as evidenced by the dot plots for dry weight (Figure 6.11).

A suitable recovery media should be chosen according to the objective of the experiment. If the aim of experiment is to investigate rapidly seed germination rate and not on the further development of the seedling, then filter paper will suit this purpose. Filter paper substrate is more economical and less laborious compared to the other two options used. MS medium is suitable for *in vitro* germination for tissue culture purpose, micro-propagation and cryopreservation. The advantage of *in vitro* medium is that it allows manipulation of exogenous hormones or plant growth regulators to promote growth of selected explants. This could be important to induce germination of difficult to germinate seeds, overcome certain types of dormancy, in embryo rescue techniques and micro-propagation of vegetative tissues (Yamaguchi and Kamiya, 2002; Koormeef *et al.*, 2002). Soil substrate is appropriate for assessing further development of the seedling and also for subsequent planting in the nursery.

6.5.6 Conclusions

C. siamea seeds were successfully cryopreserved with high germination and the best treatment combination was 9hrs desiccation, slow-rapid warming and in filter paper medium which gave the highest viability of 90% (Table 6.6). Overall, it was found that drying time and germination medium are the most important factors for cryopreservation of *C. siamea* seeds. Drying time defines the optimum moisture content required to achieve post-cryopreservation survival. Filter paper is recommended as the suitable germination media since only immediate viability after cryopreservation was assessed and the seedlings were not transferred *in vivo*. The full factorial, fractional and Taguchi experiments gave almost the same results.

Having successfully applied efficient experimental designs (Taguchi and factorial) for cryopreservation of an orthodox seed, the next chapter will investigate the application of these for cryopreservation experiments of two tropical intermediate species.

Table 6.13 Summary of key findings for *C. speciosa* cryopreservation (3^3) experiment

Experimental design	Full factorial (3^3)	Fractional factorial (3^{3-1})	Taguchi L_{27}	Taguchi L_9
Sprouting day				
Significant main effect				
Drying time	S, p=0.013	NS	NS	NS
Warming rate	NS	NS	NS	NS
MS medium compared to filter paper	S, p=0.000	S, p=0.004	S, p=0.000	S, p=0.002
Soil substrate compare to filter paper	S, p=0.000	S, p=0.001	S, p=0.000	S, p=0.000
Significant interaction				
Drying time*warming rate	S, (p=0.000)	-	NS	-
Drying time*MS medium	S, (p=0.000)	-	NS	-
Drying time*soil substrate	S, (p=0.001)	-	NS	-
Warming rate*soil substrate	S, (p=0.030)	-	NS	-
Spearman's rank correlation coefficient, r_s,				
Observed mean versus observed SNR	1.000 (p<0.05)	-	1.000 (p<0.05)	-
Observed mean versus fitted mean	-	0.938 (p<0.05)	-	-
Observed SNR versus fitted SNR	-	-	-	0.938 (p<0.05)
Fitted mean versus fitted SNR	-	1.000 (p<0.05)	-	1.000 (p<0.05)
Shoot to root ratio				
Significant main effect				
Drying time	S, p=0.000	NS	NS	NS
Warming rate	NS	NS	NS	NS
MS medium compared to filter paper	S, p=0.000	S, p=0.006	S, p=0.000	S, p=0.001
Soil substrate compared to filter paper	S, p=0.000	S, p=0.002	S, p=0.000	S, p=0.001
Significant interaction				
Drying time*warming rate	S (p=0.011)	-	NS	-
Drying time*MS medium	S (p=0.003)	-	NS	-
Spearman's rank correlation coefficient, r_s,				
Observed mean versus observed SNR	1.000 (p<0.05)	-	1.000(p<0.05)	-
Observed mean versus fitted mean	-	0.911 (p<0.05)	-	-
Observed SNR versus fitted SNR	-	-	-	0.745 (p<0.05)
Fitted mean versus fitted SNR	-	0.472 (p=0.013)	-	0.472 (p=0.013)

Dry weight				
Significant main effects and interactions	No significant main effect or interaction noted	No significant main effect noted	No significant main effect or interaction noted	No significant main effect noted
Spearman's rank correlation coefficient, r_s, **Observed mean versus observed SNR**	0.955 ($p<0.05$)	-	0.955 ($p<0.05$)	-

NS= Not significant, S= Significant, parentheses show levels associated with better treatments.

Table 6.14 Summary results for *C. siamea* cryopreservation experiment by recovery media

	MS medium	**Soil substrate**	**Filter paper**
Sprouting day			
Significant main effect			
Drying time	S(p=0.000)	S(p=0.000)	NS
Warming rate	NS	NS	NS
Drying time^2	NS	NS	NS
Warming rate^2	S(p=0.000)	S(p=0.000)	NS
Significant interaction			
Drying time*warming rate	S(p=0.003)	S(p=0.039)	NS
Spearman's rank correlation coefficient, r_s			
Observed mean Vs observed SNR	1.000, p=0.000	1.000, p=0.000	0.996, p=0.000
Shoot to root ratio			
Significant main effect			
Drying time	NS	S(p=0.002)	S(p=0.000)
Warming rate	NS	NS	NS
Drying time^2	S(p=0.012)	S(p=0.027)	S(p=0.000)
Warming rate^2	NS	S(p=0.006)	S(p=0.000)
Significant interaction			
Drying time*warming rate	NS	NS	S(p=0.000)
Spearman's rank correlation coefficient, r_s			
Observed mean Vs observed SNR	0.933, p=0.000	0.983, p=0.000	1.000
Dry weight			
Significant main effect			
Drying time	NS	NS	NS
Warming rate	NS	NS	NS
Drying time^2	NS	NS	NS
Warming rate^2	S (p=0.008)	NS	S(p=0.047)
Significant interaction			
Drying time*warming rate	S(p=0.008)	S(p=0.034)	S(p=0.04)
Spearman's rank correlation coefficient, r_s,			
Observed mean Vs observed SNR	0.817, p=0.007	0.983, p=0.000	0.997, p=0.000

CHAPTER 7

CRYPRESERVATION PROTOCOL DEVELOPMENT FOR TROPICAL FOREST TREE INTERMEDIATE SEEDS

7.1 Introduction

The successful application of the Taguchi technique for orthodox seeds was achieved in Chapter six (Sections 6.4 and 6.5). Thus, in this chapter, its application is now investigated for the cryopreservation of intermediate seeds. The aim is to develop optimised cryopreservation protocols for selected intermediate seeds and their isolated embryos, and applying desiccation and vitrification strategies while sacrificing minimal germplasm.

Intermediate seeds are more difficult to cryopreserve than orthodox seeds (Engelmann, 2004). They are relatively desiccation tolerant, but do not survive the extent of water loss naturally withstood by orthodox types (Berjak and Pammenter, 2004). Therefore, considerable care is needed when applying cryoprotection treatments. However, the successful cryopreservation of intermediate embryonic axes has now been reported using simple desiccation techniques to more advanced encapsulation-dehydration and vitrification protocols (Hor *et al.*, 2005; Dussert *et al.*, 2001; Engelmann, 2004).

This chapter will report three experiments carried out on two tropical intermediate forest tree species namely *Koompassia malaccensis* and *Sterculia cordata*. *K. malaccensis* exhibits an orthodox-intermediate seed storage physiology (Flynn *et al.*, 2004) and *S. cordata* seeds exhibit an intermediate-recalcitrant seed storage behaviour, these seeds are currently stored at partially dehydrated moisture contents at the temperature of 12 to 20°C after dispersal (Seed Technology Lab. FRIM unpublished data). However, their viability is maintained only for few weeks and longevity of the stored seeds is inversely related to their moisture content and storage temperature. Therefore, the experimental parameters selected for each species were different. All three experiments were designed and carried out with mixed-level factors since this gave the flexibility to add a preferred numbers of levels for selected factors. Therefore the experiments conducted were designed to give as much information as possible within as short time frame possible.

7.2 Cryopreservation of *Koompassia malaccensis* Seeds Using Desiccation

7.2.1 Introduction

Koompassia malaccensis (locally known as kempas) belongs to the family Leguminosae and is widely distributed in Southern Thailand, Peninsular Malaysia, Sumatra and Borneo (Putz, 1978). Kempas is the third, commonest big tree in Peninsular Malaysia. The tree is up to 60m tall, with a columnar bole which is on average 60cm in diameter but sometimes up to 210cm (Ser, 1981). Kempas is listed as one of the major commercial trees in South East Asia (Soerianegara and Lemmens, 1994) and it produces a medium-heavy timber, which is used for heavy construction purposes such as buildings, ships, beams and railway sleepers (Balan Menon, 1986).

Kempas fruits have a papery wing; the seed is flattened, not protected by a hard coat (Figure 7.1) and it is naturally dispersed by wind. The seeds are conventionally stored at a temperature of 12-20°C. However, viability declines over a long storage period (Satohiko, 1980) and they are also susceptible to fungus infection. Owing to these various problems and limitations with conventional storage, it is necessary to investigate an alternative or complementary storage method for long-term storage.

Figure 7.1 Seeds of *Koompassia malaccensis*

Cryopreservation using liquid nitrogen at −196°C appears to be a logical choice for the long-term conservation of *K. malaccensis* seeds germplasm. Desiccation has been applied to several large-seeded tree species previously (Pence, 1990), and was therefore examined for *K. malaccensis* seeds. This technique is mainly used with zygotic embryos or embryonic axes excised from seeds, and has been applied to embryos of several recalcitrant and intermediate seeds (Engelmann, 2004).

In this experiment, seeds and excised embryos were compared for cryo-storage, because seeds with intermediate-recalcitrant behaviour often have relatively large seeds and high moisture contents which pose problems for cryo-storage. Furthermore, intermediate-recalcitrant embryos are reported to be more tolerant to desiccation and low temperatures compared to whole seeds and, together with their smaller size are more technically feasible for cryo-conservation (Wesley-Smith *et al.*, 2001). Furthermore, embryo rescue methods under *in vitro* conditions assist in the reduction of microbial contamination especially seed-borne fungi (Calistru *et al.*, 2000).

As the procedures involve *in vitro* manipulations, tissue culture parameters were also explored. Different concentrations of endogenous hormone of benzyl amino purine (BAP) in recovery media were also investigated. This is because the establishment of a suitable recovery medium is essential, particularly when excised embryos are used for germplasm storage. It is an important prerequisite that a suitable medium supports regeneration after cryopreservation.

The experiment was therefore designed with three factors with mixed-levels as a 4 x 2^2 experiment:

A: Explant; 1) whole seed dried for 8 hr,
2) whole seed dried for 4 hr,
3) embryo axes dried for 2.5 hr and
4) embryo axes dried for 5 hr

B: LN storage; 1) -LN and 2) +LN

C: BAP level in recovery media; 1) 0.5 g/l and 2) 1.0 g/l

7.2.2 Materials and methods

7.2.2.1 Seed material and processing

Seeds were collected from mother trees within the FRIM campus and dried in the laboratory at room temperature (25-28°C) for two days after which they were washed in running tap water, surface sterilized with (10% v/v hypochlorite solution, Domestos, Lever Bros.) for 10 minutes, 0.3% (w/v) boric acid and rinsed once in 50% (v/v) ethanol for one minute, and then rinsed three times in sterile water. The embryonic axes were excised aseptically using a scalpel in a laminar airflow cabinet. Sterilized seeds and embryos were arranged in a single layer in sterile Petri dishes and placed in the air current of laminar airflow cabinet for the required period. The room temperature and relative humidity during seed desiccation were 23 $\pm$ 2°C and 55 $\pm$ 5% respectively.

7.2.2.2 Cryopreservation

After the required desiccation period, half of the seeds and embryos were placed in sterile 2ml cryo-vials (Nalgene, Sigma) and cooled rapidly by direct immersion in liquid nitrogen. Both the seeds and the embryos were rapidly warmed by direct plunging and swirled in a water bath at 38 + 2°C for 10-15 minutes.

7.2.2.3 Post-cryopreservation recovery assessments

For both the seeds and embryos, *in vitro* germination in Murashige and Skoog (1962) medium (MS) supplemented with 30g/l sucrose (Duchefa Biochemie) and 5.5g/l Plant Agar (Duchefa Biochemie) was applied in combination with BAP. The seeds and embryos were cultured in a temperature-controlled growth room at 26°C with a 12 hours photoperiod. The development of each seed from sprouting to germination was observed daily over 10 days. Assessments of seed and embryo moisture content, germination, shoot to root ratio and seedlings dry weight were carried out as described for *C. siamea* seeds in Section 6.4.3.

7.2.2.4 Data analysis

Twenty embryos were used in each treatment combination. Four experimental designs namely, full factorial, ½ fractional factorial and Taguchi L_{16} and L_8 (Table 7.1) were compared for data analysis using ANOVA and SNR and the ranking for best treatments was carried out as described in Section 5.5.

Table 7.1 Experimental designs and their selected treatment combinations

Run	Explant	LN	BAP (g/l)	Full factorial experiment	Taguchi full experiment L_{16}	Half factorial / Taguchi L_8
1	Seed/8h	-	0.5	**Y**	**Y**	**Y**
2	Seed/8h	-	1.0	**Y**	**Y**	
3	1seed/8h	+	0.5	**Y**	**Y**	
4	Seed/8h	+	1.0	**Y**	**Y**	**Y**
5	Seed/4h	-	0.5	**Y**	**Y**	**Y**
6	Seed/4h	-	1.0	**Y**	**Y**	
7	Seed/4h	+	0.5	**Y**	**Y**	
8	seed/4h	+	1.0	**Y**	**Y**	**Y**
9	Embryo/8h	-	0.5	**Y**	**Y**	**Y**
10	Embryo/8h	-	1.0	**Y**	**Y**	**Y**
11	Embryo/8h	+	0.5	**Y**	**Y**	
12	Embryo/8h	+	1.0	**Y**	**Y**	
13	Embryo4h	-	0.5	**Y**	**Y**	
14	Embryo/4h	-	1.0	**Y**	**Y**	**Y**
15	Embryo/4h	+	0.5	**Y**	**Y**	**Y**
16	Embryo/4h	+	1.0	**Y**	**Y**	

7.2.3 Results

Kempas seeds had an initial moisture content of 20% with the excised embryo having slightly higher moisture of 27 % on a fresh weight basis. The moisture contents of seeds dried to four and eight hours and the embryos dried for 2.5 and 5hr and their germination percentage after desiccation compared to the initial stage is given in Figure 7.2.

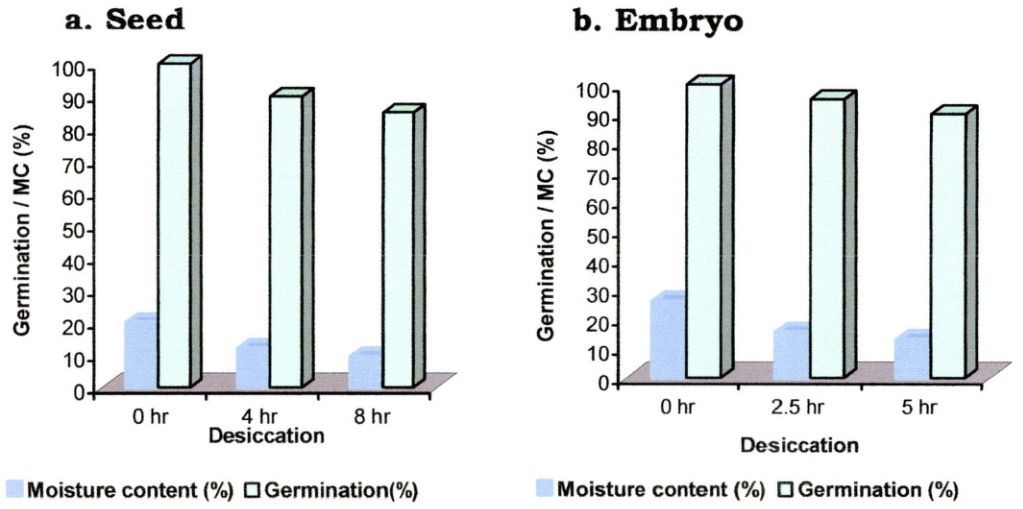


Figure 7.2. Percentage moisture content (fresh weight basis) and germination of kempas seeds after 4, 8 hr desiccation (a) and for kempas embryos after 2.5, 5 hr desiccation, (b) respectively as compared to initial moisture content and % germination.

7.2.3.1 Germination day

ANOVA and Taguchi analyses of full experiments showed that use of different explants have a significant effect ($p<0.000$) on germination day. Seeds dried for 4hr (moisture content = 12%) germinated more rapidly compared to seeds dried for 8hr (moisture content = 9.5%) and seeds overall germinated rapidly compared to embryos (Figure 7.3).

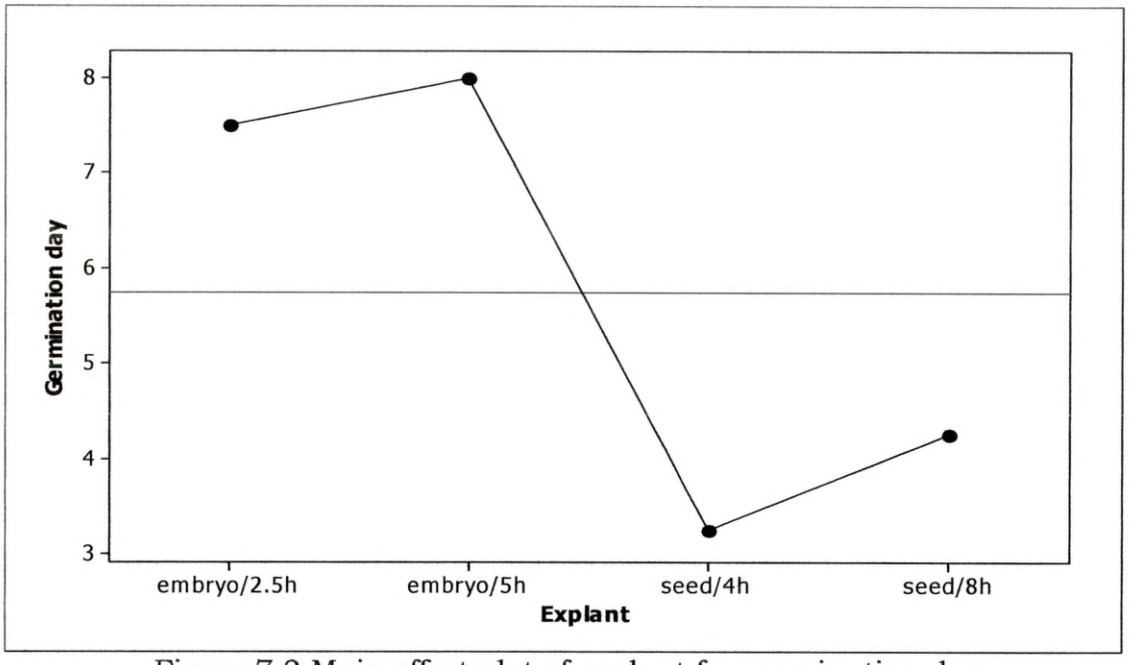


Figure 7.3 Main effect plot of explant for germination day

The Spearman's rank correlation coefficient was very high (r_s = 0.999, p<0.001) for mean and SNR showing that both analyses produce similar conclusions. Table 7.2 shows the five best treatments selected for smallest germination day by using ANOVA.

Table 7.2. Five best treatments selected for the smallest germination day using the mean, their corresponding SNR_{Small} and ranks in the full experiment

Explant	**LN**	**BAP (g/l)**	**Mean**	**Rank**	**SNR**	**rank**
Seed/4hr	-LN	0.5	3.0625	1	-9.9178	1
Seed/4hr	-LN	0.5	3.2353	2	-10.2723	2
Seed/4hr	-LN	1.0	3.3889	3	-10.8912	3
Seed/8hr	+LN	0.5	3.4444	4	-10.9884	4
Seed/8hr	-LN	0.5	3.5789	5	-11.3287	6

The half fractional replicates of both factorial and Taguchi experiment analyses also produced exactly same results as the full experiments (Table 7.5).

7.2.3.2 Shoot to root ratio

ANOVA of the full factorial experiment showed significant effects of explant ($p < 0.001$) and BAP ($p < 0.001$) on shoot to root ratio. Seedling obtained from embryos tended to have shoot to root ratio closer to one compared to seeds (Figure 7.4). The seedlings on 0.5g/l BAP had shoot to root ratio closer to one compared to those on 1.0g/l BAP in recovery medium (Figure 7.4). There were also highly significant two-way interactions ($p < 0.001$) between explant and LN storage and between explant and BAP (Table 7.5; Figure 7.5). However, Taguchi full experiment analysis did not show any significant main effects or interactions. Half fractional factorial and Taguchi experiments analyses showed exactly the same conclusions as the full experiment. Spearman's rank correlation between treatments for means closest to one and $SNR_{Nominal}$ was $r_s = 0.725$ ($p < 0.05$). This shows a significant but not exact agreement for both methodologies (Table 7.3).

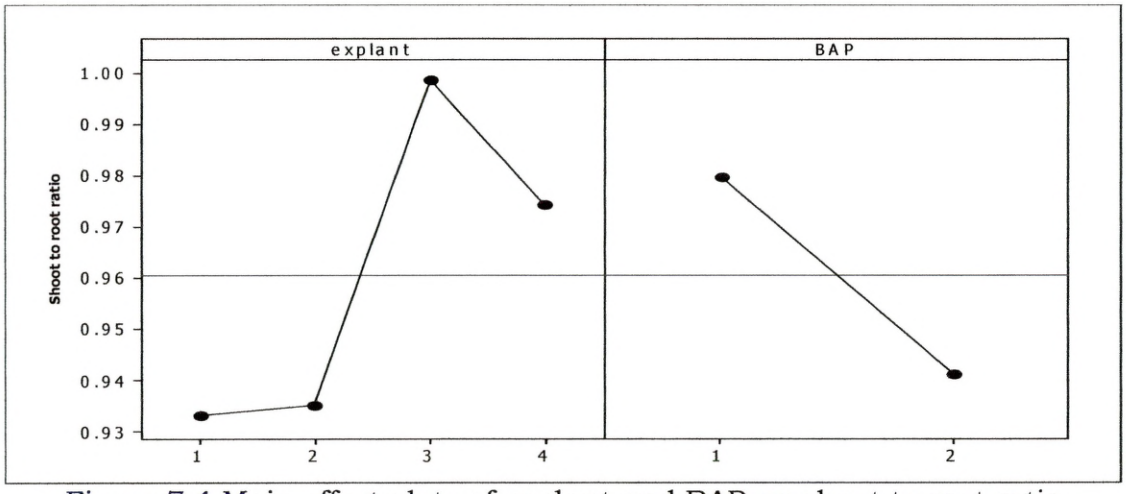


Figure 7.4 Main effect plots of explant and BAP on shoot to root ratio

Note: Explant level 1= seed/4h, 2 = seed/8h, 3 = embryo 2.5h, 4 = embryo 5h; BAP level 1 = 0.5 g/l, 2 = 1.0 g/l.

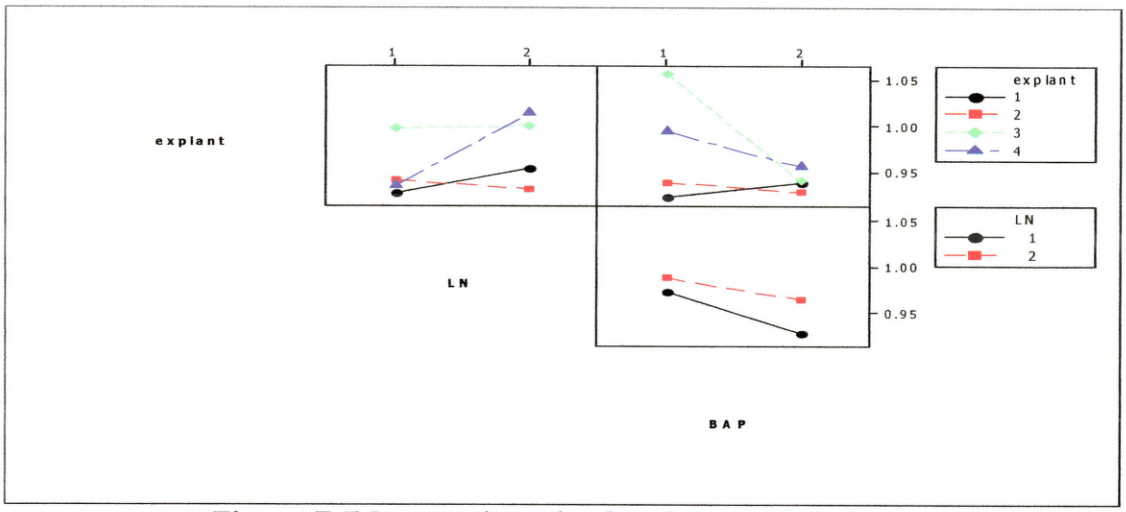


Figure 7.5 Interaction plot for shoot to root ratio

Note: Explant level 1= seed/4h, 2 = seed/8h, 3 = embryo 2.5h, 4 = embryo 5h; LN level 1 = -LN, 2 = +LN.

Table 7.3 Five best treatments selected for shoot to root ratio closer to one using the mean, their corresponding $SNR_{Nominal}$ and ranks in the full experiment

Explant	**LN**	**BAP (g/l)**	**Mean**	**Rank**	**SNR**	**Rank**
Embryo/2.5hr	-LN	0.5	1.0021	1	13.8179	18
Embryo/2.5hr	-LN	1.0	1.0065	2.5	15.5370	8
Embryo/5hr	-LN	1.0	1.0065	2.5	16.3723	4
Seed/8hr	-LN	0.5	0.9917	4	15.5830	7
Embryo/2.5hr	+LN	0.5	1.0095	5	15.0147	12

Treatment combination in rank one for mean was in rank 18 for SNR and the treatment combination in rank one for SNR was in rank 11.5 for mean. The dot plots shows that the treatment combination in rank one for SNR is closely distributed around one, compared to treatment combination with the best mean closest to one (1.0021) as shown in Figure 7.6.

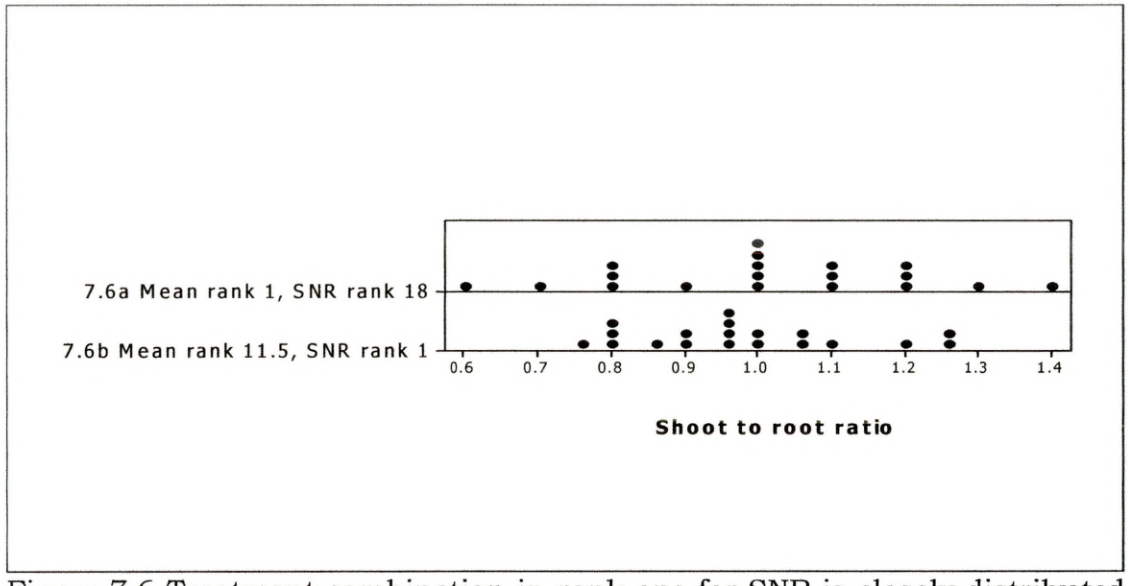


Figure 7.6 Treatment combination in rank one for SNR is closely distributed around target [b], compared to treatment combination with the mean closest to one [a]

7.2.3.3 Dry weight

ANOVA and SNR analyses showed that explant had a highly significant effect on seedling dry weight ($p < 0.001$). Seedlings obtained from seeds tended to have greater dry weight compared to those obtained from embryos (Figure 7.7). No significant two-way interaction was noted in either analysis. There was a significant Spearman's rank correlation ($r_s = 0.986$, $p<0.05$) between mean and SNR. The five best treatments for largest dry weight using mean and their corresponding SNR_{Large} are shown in Table 7.4.

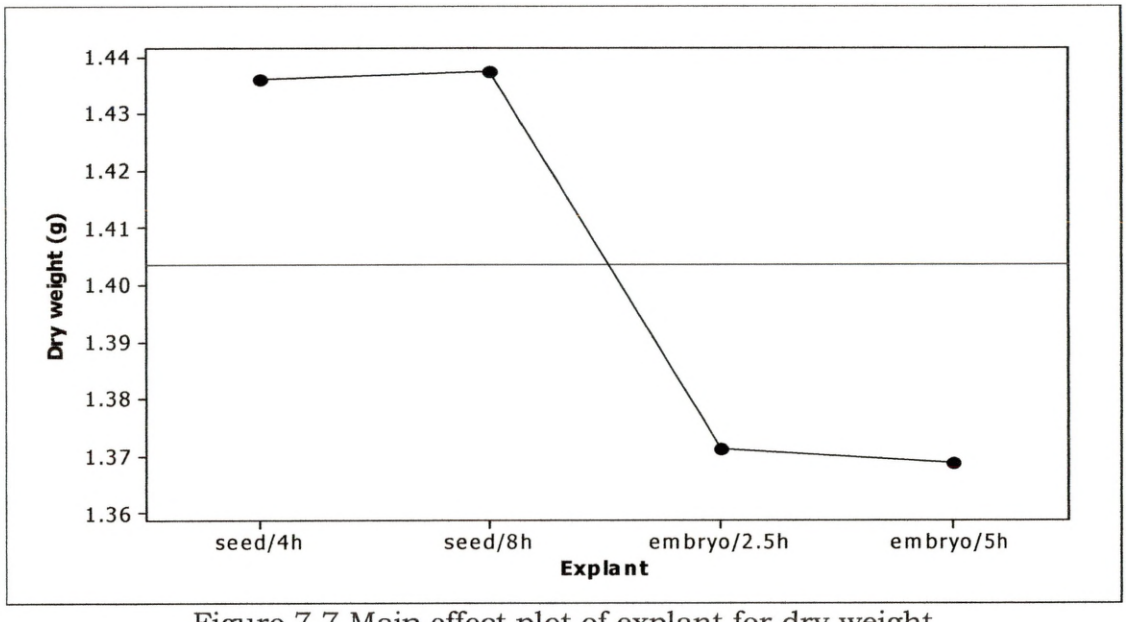


Figure 7.7 Main effect plot of explant for dry weight

Table 7.4 Five best treatments selected for largest dry weight using the mean, their corresponding SNR_{Large} and ranks in the full experiment

Explant	**LN**	**BAP (g/l)**	**Mean**	**rank**	**SNR**	**rank**
Seed/4hr	+LN	1.0	1.55074	1	-5.3128	1
Seed/4hr	+LN	0.5	1.51522	2	-5.9404	2
Seed/8hr	+LN	1.0	1.49039	3	-6.7543	6
Seed/8hr	+LN	1.0	1.49016	4	-6.8500	7
Seed/8hr	-LN	0.5	1.48732	5	-6.7410	4

Table 7.5 gives the summary of the conclusions for significant main effects, two-way interactions and Spearman's rank correlation between ranks of observed mean versus observed SNR, observed mean versus fitted mean, observed SNR versus fitted SNR and fitted mean versus fitted SNR for all three response variable obtained using different experimental designs.

Table 7.5 Summary results for cryopreservation using desiccation method ($4x2^2$) experiment For *K. malaccensis*

Experimental design → **Observations ↓**	Full Factorial	½ Fractional Factorial	Taguchi Full L_{16}	Taguchi L_8
Germination Day				
Significant main effect				
Explant	S***	S***	S***	S***
LN storage	S**	S*	S*	S*
BAP hormone	S**	S*	S*	S*
Significant Interactions				
Explant*LN	NS	NS	NS	NS
Spearman's rank correlation coef .r_s,				
Obs. mean vs. Obs. SNR	0.999***	-	0.999***	-
Obs. mean vs. fitted mean	-	0.916***	-	-
Obs. SNR vs. fitted SNR	-	-	-	0.903***
Fitted mean vs. fitted SNR	-	0.982**	-	0.982**
Shoot to root ratio				
Significant main effect				
Explant	S***	S**	NS	NS
LN storage	NS	NS	NS	NS
BAP hormone	S***	NS	NS	NS
Significant Interactions				
Explant*LN	S***	NS	NS	NS
Explant*BAP	S**	S***	NS	NS
Spearman's rank correlation coefficient, r_s,				
Obs. mean vs. Obs. SNR	0.725***	-	0.725***	-
Obs. mean vs. fitted mean	-	0.383*	-	-
Obs. SNR vs. fitted SNR	-	-	-	0.194(NS)
Fitted mean vs. fitted SNR	-	0.787***	-	0.787***
Dry weight				
Significant main effect				
Explant	S***	S***	S***	S**
LN storage	NS	NS	NS	NS
BAP hormone	NS	NS	NS	NS
Spearman's rank correlation coefficient, r_s,				
Obs. mean vs. Obs. SNR	0.986***	-	0.986***	-
Obs. mean vs. fitted mean	-	0.696***	-	-
Obs. SNR vs. fitted SNR	-	-	-	0.684***
Fitted mean vs. fitted SNR	-	0.952***	-	0.952***

NS=Not significant, S= Significant at $p<0.05$ for *, $p<0.01$ for ** and $p<0.001$ for ***; (-) = not relevant

7.2.4 Discussion

7.2.4.1 Post-cryopreservation recovery

A successful cryopreservation protocol was developed for whole seeds and zygotic embryos of *K. malaccensis* species for the first time in this study. *K. malaccensis* seeds and embryos had an initial moisture content of approximately 20 and 27% respectively. Desiccation before cryopreservation is necessary to avoid the formation of intracellular ice crystals (Mazur, 2004). A longer desiccation time was selected for whole seeds (4 and 8 h) compared to excised embryos (2.5 and 5 h) since Pritchard *et al.* (1995), reported that the embryo tissues of *Araucaria hunsteinii* dried at a rate about 200 times faster when isolated compared to those dried within intact seeds.

During cryopreservation, decreasing moisture content reduces the likelihood of freezing of intermediate-recalcitrant embryos at sub-freezing temperatures (Hor *et al.*, 2005; Krishnapillay, 2000). However, extensive dehydration results in embryo death (Nadarajan, 1999; Engelmann, 2004). A correlation has been proposed between intolerance to drying of embryo tissues and the removal of matrix-bound water as determined by pressure-volume analysis (Finch-Savage, 1992).

In this experiment, it was noted that seeds germinated more rapidly compared to embryonic axes. This may be due to developmental factors probably because cotyledons supply nutrients required for seedling development and germination which was triggered immediately on recovery media. In the case of excised embryonic axes, nutrient supply cotyledons is not present and development is dependent on external nutrients and plant growth regulators. This may therefore, cause a delay in germination in the embryonic axes. According to Pence (1995), culture medium should be optimised to detect possible damage due to desiccation and freezing. Garcia-Martin *et al.* (2001), reported that embryos of *Quercus suber* were not able to germinate without external addition of sucrose. Furthermore, Gonzalez-Benito *et al.*, (2002) reported that shoot elongation of *Quercus suber* and *Q. ilex* were favoured by the use of $2mgl^{-1}$ BAP without affecting root

development. These observations suggest that careful manipulation of recovery medium is important for germination and further development of excised embryos.

Another factor that could contribute to the delay in germination for excised embryos is the mechanical damage caused by dissection. *Coffea liberica* seeds with endocarp intact survived cryopreservation better than those without, suggesting that perhaps the endocarp gives protection or dissection damage could have occurred and affected the viability of those excised embryos (Normah and Vengadasalam, 1992). Goveia *et al.* (2004), noted that root growth was proportionally depressed relative to the increasing amount of cotyledonery tissue attached to the axis in *Trichilia dregeana.* They also suggested that this phenomenon is due to leakage of an inhibitory or toxic substance(s) from the cut surfaces of the cotyledonery tissues into the germination medium. There could also be an oxidative burst associated with, and part of the wounding response as demonstrated in wheat roots (Minibayeva *et al.*, 2003). Minibayeva *et al.*, (ibid) also reported a significant increase in the production of extra-cellular peroxidases associated with the formation of superoxide radicals when the roots were excised from the wheat seedlings.

In this experiment, excised embryos tended to have shoot to root ratio closer to one, compared to seeds (Figure 7.4). This may be because seeds in nature do not require additional growth regulators for further development of the seedling. When additional BAP is supplied, together with nutrients from intact cotyledons, vigorous growth with extended roots is noted. Excised embryos may require an exogenous hormone supply which when applied in recovery media may moderate the equivalent growth of shoots and roots. In *Trichilia dregeana* species, shoot length increased with the amount of cotyledonery tissue attached to the excised embryos (Goveia *et al.,* 2004).

In this experiment, seedlings developed from the seeds tended to have greater dry weight compared to those from embryos (Figure 7.7). This may be because optimized nutrients are made available in seeds and cotyledons, which contribute to seedling development. Since excised embryos are dependent upon supplemented exogenous nutrients that may not be

optimised to simulate those of the intact seeds, this could result in seedling lower dry weights. These results show that for seed cryopreservation in *K. malaccensis*, it is not necessary to apply external growth regulators and nutrients in the recovery medium. However, for excised embryo cryopreservation, additional growth regulators and nutrients are recommended.

As expected, seeds and embryos that were not stored in LN, germinated more rapidly compared to those stored in LN. This may be due to cryo-injury, impairment of function as evidenced by a lag phase in germination in which metabolic activity is limited and requires a period of time to recover. Metabolic processes return to normal rates, eventually and the seeds or embryos germinate as usual. Cryostorage, generally necessitates the use of excised zygotic embryonic axes that are sufficiently small to be rapidly partially dehydrated (Berjak *et al.*, 1999). However, other important contributing factors are the maturity stage of the embryos. In practice, the axis must be sufficiently mature to facilitate its ongoing development *in vitro*, which may necessitate a compromise between the optimal explant sizes for cryopreservation and subsequent plant development (Goveia *et al.*, 2004).

7.2.4.2 Comparison of experimental designs

Six experimental designs were compared for the cryopreservation of kempas seeds and embryos (Table 7.5). Significant main effects and two-way interactions for germination day and dry weight assessments were identical both in full and half replicated factorial and Taguchi experiments. However, for shoot to root ratio, all Taguchi experiments showed completely different results compared to factorial experiments. This is an example where using both ANOVA and SNR analyses could give very different conclusions in identifying significant main effects and interactions.

For the shoot to root ratio, the ranking for the best treatment combinations obtained using ANOVA and SNR analyses were similar as the Spearman's correlation coefficient between observed mean and observed SNR was highly significant (r_s = 0.725, $p<0.05$). The dot plot for this

response variable showed that the best treatments chosen using SNR are more consistent compared to the best treatment chosen using ANOVA (e.g. Figure 7.6). This observation was also made for shoot to root ratio and dry weight assessments in the cryopreservation experiment for orthodox seed (Section 6.4.5.1).

A comparison of the full factorial experiment with the half replicated experiment showed similar results for germination day and dry weight but not for shoot to root ratio. This observation was also true for the comparison of Taguchi full and half replicated experiments. In this experiment, half replicated experiments allowed investigation of all factors and two-way interactions. For this experiment, since main effects and all two-way interactions were regarded as important, it is possible to conduct only half fraction of the experiment and yet obtain the same conclusion. This advantage is important for kempas seeds where there is difficulty in collecting large number of seeds to conduct the full experiment due to irregular flowering and fruiting behaviour of this species (Soerianegara and Lemmens, 1994). These findings also endorse the need for designing experiments from an informed technical and species knowledge basis.

7.2.5 Summary of findings

1. Both kempas seeds and embryos were successfully cryopreserved for the first time using desiccation technique with high survival (>80%) after cryopreservation. This study showed that cryopreservation could be a suitable alternative method for the long-term storage and conservation of *K. malaccensis* germplasm without effecting storage viability.

2. Seeds were more readily germinated after cryopreservation compared to embryos.

3. Seedlings developed from whole seeds tended to have greater dry weight compared to those developed from excised embryos.

4. Seedlings developed from excised embryos have shoot to root ratios closer to one compared to those from whole seeds.

5. The Taguchi methodology was shown to be successful in this *K. malaccensis* cryopreservation experiment, providing more consistent conclusions compared with the factorial experiment.

6. Where there is a different conclusion noted using ANOVA and SNR (e.g. shoot to root ratio), the Taguchi methodology provides more consistent and robust results.

7. This experiment again confirmed that for this application, full experiments are not needed in practice. It is recommended to use half replicated experiments for both factorial with resolution of at least IV and Taguchi experiments since they allow investigation of all main effects and two-way interactions.

8. It is important to combine a practical /technical pre-knowledge of seed physiology and behaviour with the experimental design protocols.

7.2.6 Further progression

Seeds and embryos of *K. malaccensis* showed different responses to liquid nitrogen storage. For future experiments using larger intermediate and recalcitrant seeds, only excised embryos will be used for cryostorage since as the seed size increases, it will be more difficult to cryopreserve the whole seed. Seed physiology especially the maturity stage of seed at cryopreservation is another critical factor for success for non-orthodox seeds. Therefore, in the next section, the effect of maturity stage of an intermediate seed on cryopreservation was investigated.

7.3 Effects of Seed Development Stages, Moisture Content and Recovery Media on the Cryopreservation of *Sterculia cordata*

7.3.1 Introduction

This experiment reports, for the first time, the cryopreservation of *S. cordata* zygotic embryos, taking into consideration different cryoprotective factors as compared to previous experiments. In this experiment, only excised embryos were used due to their larger size (2cm height and 1.5cm diameter) (Figure 7.8) and high shedding moisture content of the seeds (29%). The different developmental stages of the seeds were studied for cryopreservation. Developmental stage is a very important criterion in cryopreservation especially for non-orthodox seeds (Goveia *et al.*, 2004). This is because complex physiological changes take place as the seeds mature and this includes changes in moisture content (Walters, 1999). Walters (ibid) has also shown that as embryos mature they progressively acquire tolerance to lower water potentials in discrete steps. *S. cordata* seeds which are intermediate in storage category were selected to study the effect of stage of maturity on cryopreservation. Moisture content is another important factor in cryopreservation and achieving the critical moisture content is a basic requirement in cryopreservation. Germination testing before LN storage reveals the effect of maturation stage and critical moisture content on embryo viability. Since excised embryos were used in this experiment, different levels of BAP hormone was also tested in the recovery media.

7.3.2 Species introduction

An important tropical tree species and member of the Sterculiaceae family, *Sterculia cordata* Blume is endemic in Peninsular Malaysia, Borneo, Sumatra and New Guinea and distributed throughout South-East Asia (Ng and Low, 1982). It produces lightweight softwood used for light interior constructions, packing cases, ceiling, veneer and plywood, concrete shuttering and heels of shoes. The wood is also suitable for boat building and for pulp and paper manufacture. The seeds of *S. cordata* are edible and have a high oil or fat content, which is used for frying, illumination and also in batik works (Lemmens *et al.*, 1998). Seed production of this species is

very irregular making the guaranteed availability of seeds generally difficult. This is made more problematic with the competition with small mammals (e.g. squirrels), monkeys and birds (e.g. hornbills) that feed on their fleshy cotyledons (Veevers-Carte, 1984).

The seeds are dispersed after reaching a moisture content of 26% (fresh weight basis) and they usually germinate immediately or soon after falling from the mother trees. They are currently stored at partially dehydrated moisture contents at the temperature of 12 to 20°C after dispersal. However, seed viability is maintained only for few weeks and longevity of the stored seeds is inversely related to their moisture content and storage temperature. Based on their post-harvest storage behaviour, these seeds could be classified as intermediate (Ellis *et al.*, 1990; Roberts, 1973). The seeds are also prone to fungal infection and attacks by pests, which further reduce storage viability. Therefore, under these conditions, long-term storage of these seeds is not currently possible and cryopreservation offers the only current potential solution for the long-term storage of this species.

7.3.3 Materials and methods

7.3.3.1 Seed material

Seedpods at developmental stage one (SDS 1), two (SDS 2) and three (SDS 3) were collected from mother trees within the Forest Research Institute of Malaysia (FRIM) campus. Seeds at stage three (SDS 3) were collected at their optimum maturity where they are ready to shed from their mother trees. Seeds at development stage two (SDS 2) and stage one (SDS 1) were collected at one week interval for each stage (one and two weeks before collecting seeds at stage three respectively). These intervals were set and fixed based on FRIM's monthly phenological report on this species.

Figure 7.9 shows the three different developmental stages of seeds used in this experiment. Seed moisture contents, germination test and dry weights were determined at these three different stages using the same methodologies described in Section 6.4.

Figure 7.8 *Sterculia cordata* seed pods and seeds

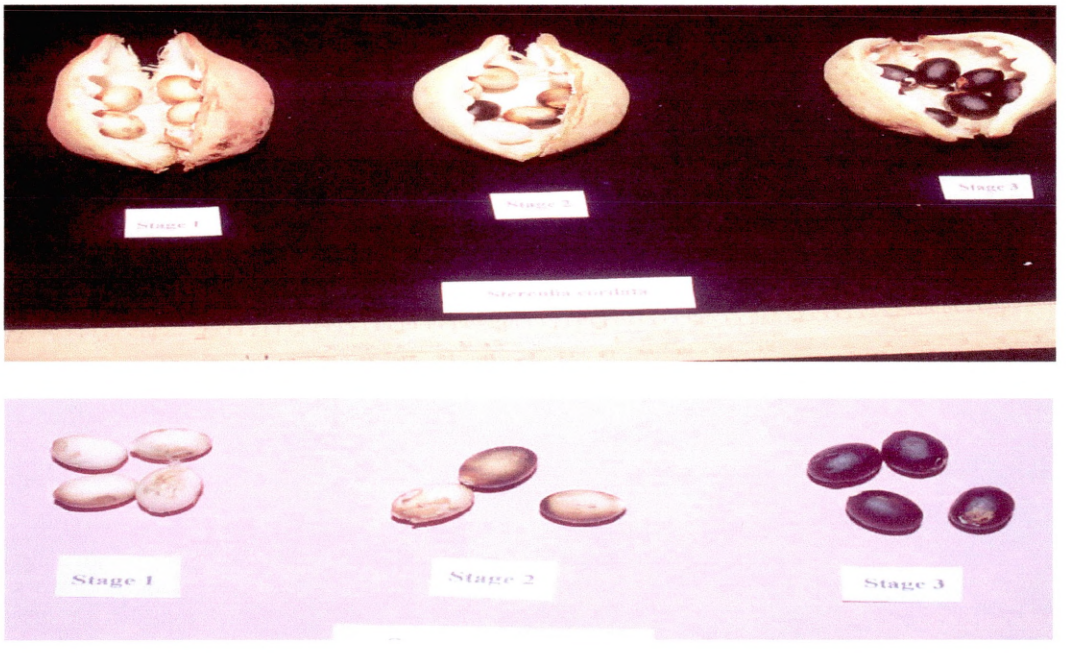


Figure 7.9 *Sterculia cordata* seeds at three different developmental stages

7.3.3.2 Seed processing and testing

Seeds were surface sterilized (10% (v/v) hypochlorite solution, Domestos, Lever Bros.) for 10 minutes, rinsed once in 30% (v/v) ethanol for one minute, and then rinsed three times in sterile water. The embryos were excised in a laminar airflow cabinet, surface sterilised with 0.3% (w/v) boric acid, rinsed once in 30% (v/v) ethanol for one minute and rinsed three times

in sterile water. Seed moisture content, germination rates and dry weights were determined at each stage, as described previously by Muthusamy *et al.* (2005).

7.3.3.3 Seed desiccation

Excised embryos were arranged in a single layer in sterile Petri dishes and placed in the air current of a laminar airflow cabinet, and desiccated for two, four and six hours at 23 ± 2°C and 55 ± 5% RH. Embryo moisture contents were determined after desiccation at each developmental stage.

7.3.3.4 Cryopreservation

After the required desiccation period, half of the embryos were placed in sterile 2ml cryo-vials (Nalgene, Sigma), and cooled rapidly by direct immersion in liquid nitrogen. They were rapidly warmed by directly plunging and swirling in a water bath at 38 + 2°C for 10-15 minutes.

7.3.3.5 Germination

Both non-cryopreserved and cryopreserved embryos were germinated in Murashige and Skoog (1962) medium (MS) supplemented with 30g/l sucrose (Duchefa Biochemie) and 5.5g/l Plant Agar (Duchefa Biochemie) and three different levels of BAP (0.5, 1.0, and 1.5 g/l), incubated in a temperature-controlled growth room at 26°C with a 12 hours photoperiod. The development of each seed from sprouting to germination was observed daily for 15 days. Germination, shoot to root ratio and seedlings dry weight before and after cryopreservation were carried as described in Muthusamy *et al.*, (2005).

7.3.3.6 Data analysis

There were two experiments in this study, the first without LN storage and second with LN storage.

Experiment without LN storage

A) SDS (level –1=SDS 1, level 0=SDS 2 and level 1=SDS 3)

B) Desiccation time (level –1=2hr, level 0=4hr and level 1=6 hr)

C) BAP (level –1=0.5g/l, level 0=1.0g/l and level 1=1.5 g/l)

This is a three cubic (3^3) experiment with three factors at three levels and designed as full factorial, one third fractional, Taguchi L_{27} and L_9 experiments (Table 7.6). The response variables chosen for this case study were; germination day, shoot to root ratio and seedling dry weight. Analysis of covariance was used for this experiment since the levels of two of the factors (desiccation time and BAP concentration) are equally spaced. Therefore, the data were analysed with desiccation time and BAP concentration as covariates and seed developmental stage as a categorical factor with SDS 1 as the base level.

Table 7.6 Experimental designs and their selected treatment combinations

Run	SDS	DT (hr)	BAP (mg/l)	3^3 / L_{27}	$3^{3-1}/L_9$
1	1	2	0.5	Y	Y
2	1	2	1.0	Y	
3	1	2	1.5	Y	
4	1	4	0.5	Y	
5	1	4	1.0	Y	Y
6	1	4	1.5	Y	
7	1	6	0.5	Y	
8	1	6	1.0	Y	
9	1	6	1.5	Y	Y
10	2	6	0.5	Y	Y
11	2	6	1.0	Y	
12	2	6	1.5	Y	
13	2	4	0.5	Y	
14	2	4	1.0	Y	Y
15	2	4	1.5	Y	
16	2	2	0.5	Y	
17	2	2	1.0	Y	
18	2	2	1.5	Y	Y
19	3	6	0.5	Y	Y
20	3	6	1.0	Y	
21	3	6	1.5	Y	
22	3	4	0.5	Y	
23	3	4	1.0	Y	Y
24	3	4	1.5	Y	
25	3	2	0.5	Y	
26	3	2	1.0	Y	
27	3	2	1.5	Y	Y

SDS=seed developmental stage, DT=drying time, Y=selected treatments

Experiment with LN storage

For the cryopreservation experiment, all three levels SDS and BAP hormone were tested. However, only two levels of desiccation time were investigated since the embryos desiccated to 6hr did not show any survival for SDS 1 in experiment without LN storage. The factors and their levels are shown below;

A) SDS (level-1=SDS 1, level 0=SDS 2 and level 1=SDS 3)
B) Desiccation time (level -1=2hr and level 1=4 hr)
C) BAP (level –1=0.5g/l, level 0=1.0g/l and level 1=1.5 g/l)

This ($2x3^2$) experiment was designed as mixed-level factorial and Taguchi L_{18} experiments (Table 7.7). This experiment could not be reduced due to limited degrees of freedom to cover all the main effects. It was analyzed with mean and the appropriate SNR as described in the previous experiments.

Table 7.7 Experimental designs and their selected treatment combinations

Run	SDS	DT (hr)	BAP (mg/l)	Full factorial and Taguchi L_{18} Experiment
1	1	2	0.5	Y
2	1	2	1.0	Y
3	1	2	1.5	Y
4	2	2	0.5	Y
5	2	2	1.0	Y
6	2	2	1.5	Y
7	3	2	0.5	Y
8	3	2	1.0	Y
9	3	2	1.5	Y
10	1	4	0.5	Y
11	1	4	1.0	Y
12	1	4	1.5	Y
13	2	4	0.5	Y
14	2	4	1.0	Y
15	2	4	1.5	Y
16	3	4	0.5	Y
17	3	4	1.0	Y
18	3	4	1.5	Y

7.3.4 Results

The moisture content and dry weight of seeds at different seed developmental stages are shown in Table 7.8. There was a decline in seed moisture content and increase in seed dry weight as the seeds matured.

Table 7.8 *S. cordata* seed moisture content, dry weight and germination percentage at three different developmental stages

Parameters	SDS 1 (mean ± sd)	SDS 2 (mean ± sd)	SDS 3 (mean ± sd)
Moisture content (%)	34.33 ± 0.58	30.0 ± 1.0	29.33 ± 0.58
Dry weight (g)	3.66 ± 0.12	3.97 ± 0.13	4.44 ± 0.08
Germination (%)	40 ± 10	86.00 ± 5.29	100 ± 0

SDS= seed developmental stage, sd=standard deviation, each observation was replicated three times

The embryos moisture content at shedding and after desiccation are shown in Table 7.9. The embryos tend to have slightly higher moisture content compared to the seeds. The embryo's moisture content also declined gradually as the seed mature.

Table 7.9 Moisture contents of *Sterculia cordata* embryos at shedding and after 2, 4 and 6 hours of desiccation

Moisture content	SDS 1 (mean ± sd)	SDS 2 (mean ± sd)	SDS 3 (mean ± sd)
Shedding / 0hr	37.606 ± 0.681	33.656 ± 0.782	30.846 ± 0.687
2 hr desiccation	31.886 ± 0.626	29.056 ± 0.657	24.544 ± 0.413
4 hr desiccation	25.608 ± 0.600	22.778 ± 0.531	19.278 ± 0.609
6 hr desiccation	21.768 ± 1.278	18.672 ± 0.863	16.494 ± 0.703

SDS= seed developmental stage, sd=standard deviation, each observation was replicated three times

7.3.4.1 Experiment without LN storage

7.3.4.1.1 Comparison of ANCOVA and SNR for full experiments

Germination day

ANCOVA showed that neither desiccation time nor BAP levels have a significant effect on germination day. It also showed that both SDS 2 ($F(1,229)=102.11$, $p=0.000$) and SDS 3 ($F(1,229)=489.06$, $p=0.000$) have

significantly different effect from SDS 1. SDS 2 decreased germination day by a mean of 1.56 days compared to SDS 1 and SDS 3 decreased germination day by 3.42 days compared to SDS 1 provided other conditions are fixed. The five best treatments selected showed SDS 3 in their combinations (Table 7.10). The same conclusions were obtained using one third fractional replicate (Table 7.13).

The Taguchi analysis also showed similar results as ANCOVA. Both SDS 2 ($F(1,22)=169.35$, $p=0.000$) and SDS 3 ($F(1,22)=1112.82$, $p=0.000$) have significantly different effect form SDS 1. SDS 2 increased the SNR values for germination day by the average of 1.86 and SDS 3 increased the SNR value for germination day by the average of 4.77 compared to SDS 1 provided other conditions are fixed. A significant two-way interaction between desiccation time and SDS 3 was also noted in Taguchi L_{27} experiment [$t(17)=2.88$, $p=0.010$].

The Spearman's rank correlation between the observed means and SNR was 0.997 ($p=0.000$), showing that both methodologies have almost exactly the same rank of treatment combinations.

Table 7.10 Five best treatment combinations selected for smallest germination day using the mean, their corresponding SNR_{Small} and ranks

Desiccation time (hr)	BAP (g/l)	Mean (day)	rank	SNR	rank
4	0.5	4.3750	2	-12.9281	1.5
4	1.5	4.3750	2	-12.9281	1.5
6	1.5	4.3750	2	-13.0374	3.0
4	1.0	4.4444	4	-13.1058	4.0
2	1.5	4.5000	5	-13.4242	5.0

Note: All the treatment combination selected showed SDS 3 as optimal

Shoot to root ratio

ANCOVA showed that desiccation time had a significant effect on shoot to root ratio [$F(1,228)=7.03$, $p=0.000$]. Seedlings developed from embryos desiccated for six and four hours tended to have shoot to root ratio closer to one compared to those developed from embryos desiccated for two hours (Figure 7.10). The analysis also showed that only SDS 2

[F(1,228)=19.74, p=0.000] had significantly different effect from SDS 1. SDS 2 decreased shoot to root ratio by the average of 0.14 (from 1.08 to 0.94) compared to SDS 1 provided other conditions are fixed. There were many 2-way interactions as shown in Table 7.13.

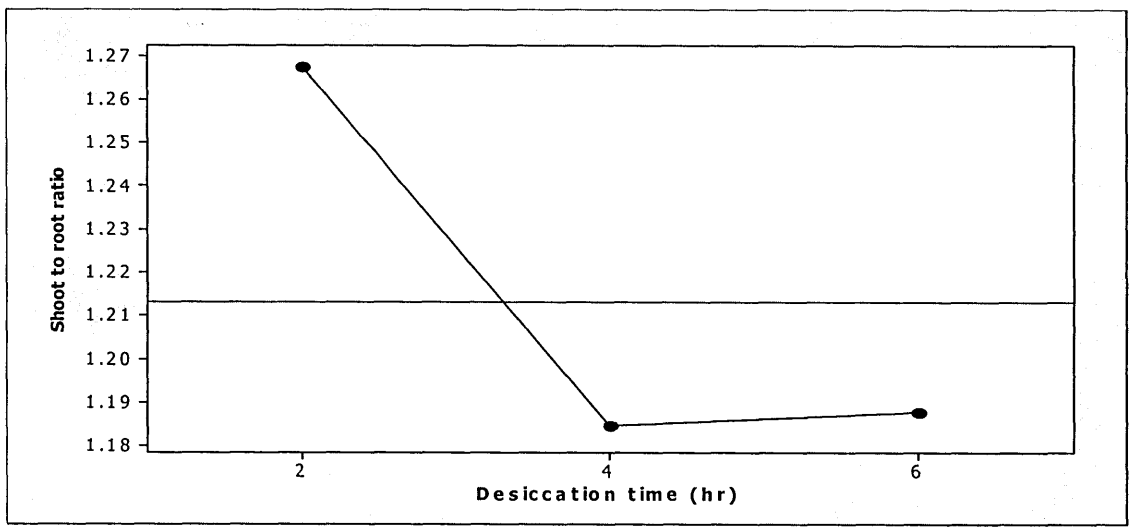


Figure 7.10 Main effect plot of desiccation time for shoot to root ratio

The Taguchi analysis also showed that only SDS 2 [F(1,22) = 6.4, p = 0.019] was significantly different from SDS 1. SDS 2 increased the SNR values for shoot to root ratio by an average of 3.62 compared to SDS 1, provided other conditions are fixed. Significant two-way interactions were noted between desiccation time and SDS 2 and also between BAP and SDS 3 (Table 7.13). The Spearman's rank correlation between observed mean and SNR was 0.945, (p = 0.000), showing that both methodologies have almost exactly the same rank of treatment combinations. Table 7.11 shows the five best treatment combinations selected for shoot to root ratio closer to one using the mean, their corresponding SNR_{Small} and their ranks.

Table 7.11 Five best treatment combinations selected for shoot to root ratio closer to one using the mean, their corresponding $SNR_{Nominal}$ and their ranks

Desiccation time (hr)	SDS	BAP (g/l)	Mean	rank	SNR	Rank
6	2	0.5	1.0625	1	15.4023	5
2	2	0.5	1.0922	2.5	17.7268	1
4	1	1.5	1.0922	2.5	17.1846	3
2	2	1.0	1.0989	4	15.8299	4
2	3	0.5	1.1050	5	17.4533	2

Dry weight

ANCOVA showed that both SDS 2 [F(1,228)=136.64, p=0.000] and SDS 3 (F(1,228)=692.44, p=0.000] have a significantly different effect from SDS 1. SDS 2 increased dry weight by an average of 0.14g compared to SDS 1 and SDS 3 increased dry weight by an average of 0.31g compared to SDS 1 provided other conditions are fixed.

The Taguchi analysis also showed that both SDS 2 [F(1,22)=74.97, p=0.000] and SDS 3 [F(1,22)=561.82, p=0.000] have significantly different effect from SDS 1. SDS 2 increased the SNR values for dry weight by an average of 1.97 and SDS 3 increased the SNR value for dry weight by an average of 5.38 compared to SDS 1 provided other conditions are fixed.

The Spearman's rank correlation between observed mean and SNR values was 0.992 (p=0.000), showing that both methodologies have almost exactly the same rank of treatment combinations. Table 7.12 shows the five best treatment combinations selected for largest using the mean, their corresponding SNR_{Large} and ranks.

Table 7.12 Five best treatment combinations selected for largest dry weight using the mean, their corresponding SNR_{Large} and ranks

Desiccation time (hr)	BAP (g/l)	Mean	rank	SNR	rank
6	1.5	0.81237	1	-1.91944	1
4	1.5	0.72384	2	-2.87732	3
6	0.5	0.69664	3	-3.25221	4
2	1.5	0.68911	4	-2.84769	2
4	1.0	0.68268	5	-3.37716	5

Note: All the treatment combination selected showed SDS 3 as optimal

7.3.4.1.2 Comparison of ANCOVA and SNR for one-third fractional experiment

This design consists of only one-third replication (i.e. nine treatment combinations) of the original experiment. For this fractional replicate it was possible to investigate all the main effects but not any interactions. ANCOVA

results for this experiment are summarised in Table 7.13. Both the one-third fractional factorial replicate and Taguchi L_9 produced similar conclusions as their full experiment counterparts apart for the shoot to root ratio response variable.

7.3.4.1.3 Overall germination before cryopreservation

The data analyses showed that only seed development stages and desiccation hour have a significant effect but not BAP hormone level in recovery media for all the response variables (Table 7.13). Therefore, overall viability as germination percentage is shown in Figure 7.11 for this experiment for seed development stage and desiccation treatment regardless of BAP level.

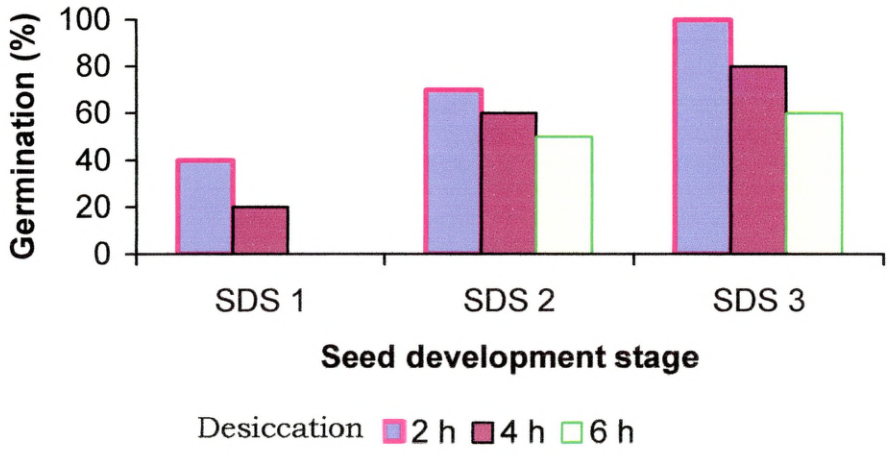


Figure 7.11 Percentage germination of *S. cordata* embryos after 2, 4 and 6 h desiccation at three seed development stages (SDS)

The percentage germination at SDS 1 after 2hr desiccation was around 40% and it declined to 20 and zero after 4 and 6hr desiccation respectively. This shows that the immature embryos are extremely sensitive to desiccation. The germination percentage was higher for SDS 2, but the embryos still showed desiccation sensitivity. The germination was higher (100%) for SDS 3 at 2hr desiccation but it also declined with desiccation period. Overall, it was found that SDS 3 was the optimum stage where *S. cordata* embryos showed highest germination.

Table 7.13 Summary of results for *S. cordata* cryo-protective experiment

Experimental designs → / **Observations ↓**	**Factorial**		**Taguchi**	
	Full	One-third Fractional	L_{27}	L_9
Germination Day				
Significant main effect				
Desiccation time (DT)	NS	NS	NS	NS
BAP	NS	NS	NS	NS
SDS 2	S***	S***	S***	S***
SDS 3	S***	S***	S***	S***
Significant Interactions				
DT*SDS 3	NS	-	S**	-
Shoot to root ratio				
Significant main effect				
Desiccation time (DT)	S***	S**	NS	NS
BAP	NS	NS	NS	NS
SDS 2	S***	S*	S**	NS
SDS 3	NS	NS	NS	NS
Significant Interactions				
DT*BAP	S*	-	NS	-
DT*SDS 2	S**	-	S**	-
DT*SDS 3	S*	-	NS	-
BAP*SDS 3	S*	-	S*	-
Dry weight				
Significant main effect				
Desiccation time (DT)	NS	S**	NS	NS
BAP	NS	S*	NS	NS
SDS 2	S***	S***	S***	S**
SDS 3	S***	S***	S***	S***
Significant Interactions				
BAP*SDS 3	S*	-	NS	-
BAP*SDS 2	S**	-	S*	-

*SDS= seed development stage, NS=Not significant, S=significant at $p<0.05$ for *, $p<0.01$ for ** and $p<0.001$ for ****

7.3.4.2 Experiment with LN storage

7.3.4.2.1 Comparison of full factorial and Taguchi experiments

Germination day

The experiment was analysed using full (2 x 3^2) factorial and Taguchi L_{18} design and results summaries are shown in Table 7.17. Seed developmental stage (SDS) showed a significant effect on embryo germination day in both factorial and Taguchi experiments ($p<0.001$). Both analyses showed that embryos at SDS 3 germinated most rapidly (Figure 7.12).

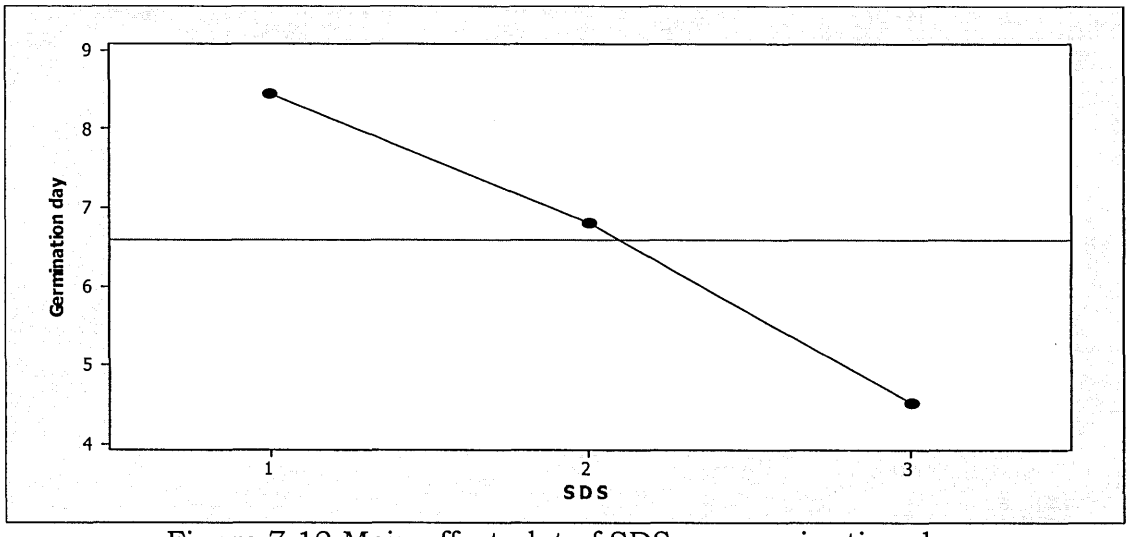


Figure 7.12 Main effect plot of SDS on germination day

There were a perfect Spearman's rank correlation between observed means and SNRs (r_s = 1.000) for germination day showing that both methodologies have exactly the same rank of treatment combinations. The five best treatment combinations selected for smallest germination day are summarised in Table 7.14.

Table 7.14 Five best treatment combinations selected for smallest germination day using the mean, their corresponding SNR_{Small} and ranks

SDS	BAP (g/l)	Mean	rank	SNR	rank
3	0.5	4.38	1	-12.928	1
3	1.0	4.44	2	-13.106	2
3	1.5	4.70	3	-13.522	3
2	1.0	6.38	4	-16.141	4
2	0.5	6.88	5	-16.824	5

Note: All the treatment combination selected showed 4hr as optimal desiccation time

Shoot to root ratio

In the shoot to root ratio assessment, SDS had a significant effect using ANOVA in the factorial experiment but the Taguchi experiment demonstrated that only the BAP level in the recovery media had a significant effect on the SNR. Whilst the factorial experiment showed no significant two-way interaction for all the response variables, the Taguchi experiment showed a significant two-way interaction between SDS and BAP for shoot to root ratio (Figure 7.13).

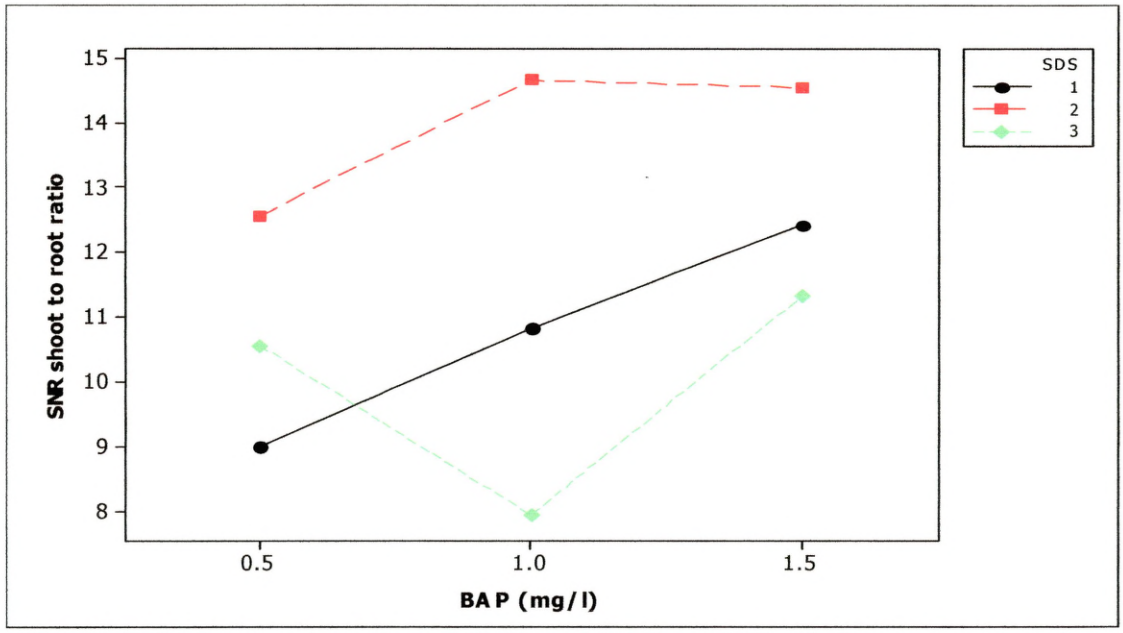


Figure 7.13 Interaction plot of SDS and BAP on SNR of shoot to root ratio

There was also a significant Spearman's rank correlation between observed means and SNR (r_s = 0.783) for shoot to root ratio, indicating that both methodologies have not exact but similar conclusions. The five best treatment combinations selected for shoot to root ratio closest to one are summarised in Table 7.15.

Table 7.15 Five best treatment combinations selected for shoot to root ratio closer to one using the mean, their corresponding $SNR_{Nominal}$ and ranks

SDS	BAP (g/l)	Mean	rank	SNR	rank
2	1.5	1.04	1	14.56	2
1	1.0	1.08	2	10.82	6
2	1.0	1.11	3	14.67	1
2	0.5	1.13	4	12.57	3
1	1.5	1.18	5	12.43	4

Note: All the treatment combination selected showed 4hr as optimal desiccation time

The treatment combination in rank one for mean was in rank two for SNR and the treatment combination in rank two for mean was in rank six for SNR and that in rank three for mean is in rank one for SNR. The dot plot for shoot to root ratio is given in Figure 7.14. This plot shows that the observations for treatment combination rank one for SNR are more closely grouped together within the range of 0.8 to 1.3.

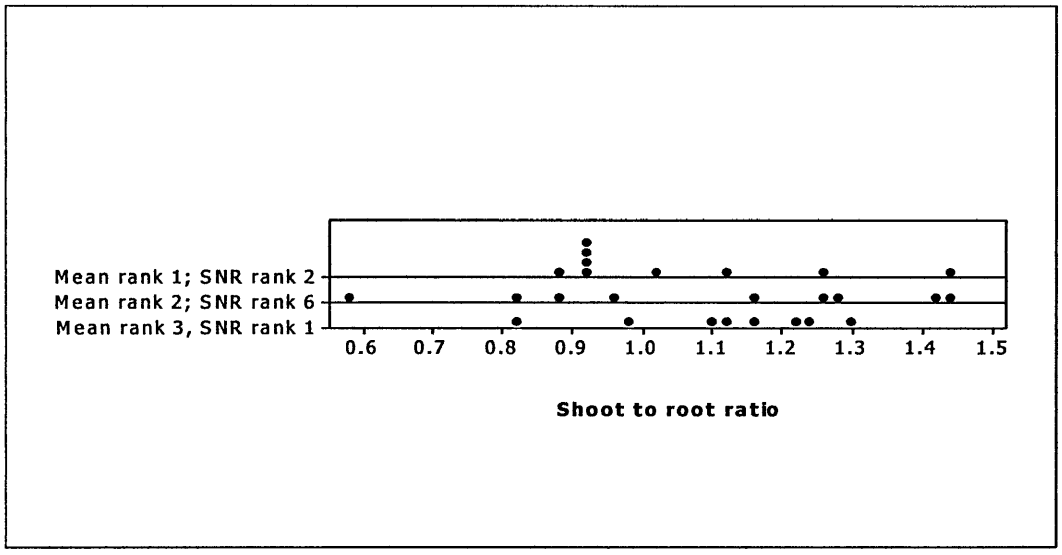


Figure 7.14 Dot plot for shoot to root ratio

Dry weight

Seed developmental stage (SDS) showed a significant effect on seedling dry weight in both factorial and Taguchi experiments ($p<0.000$). Both analyses showed that embryos at SDS 3 had a higher dry weight (Figure 7.15).

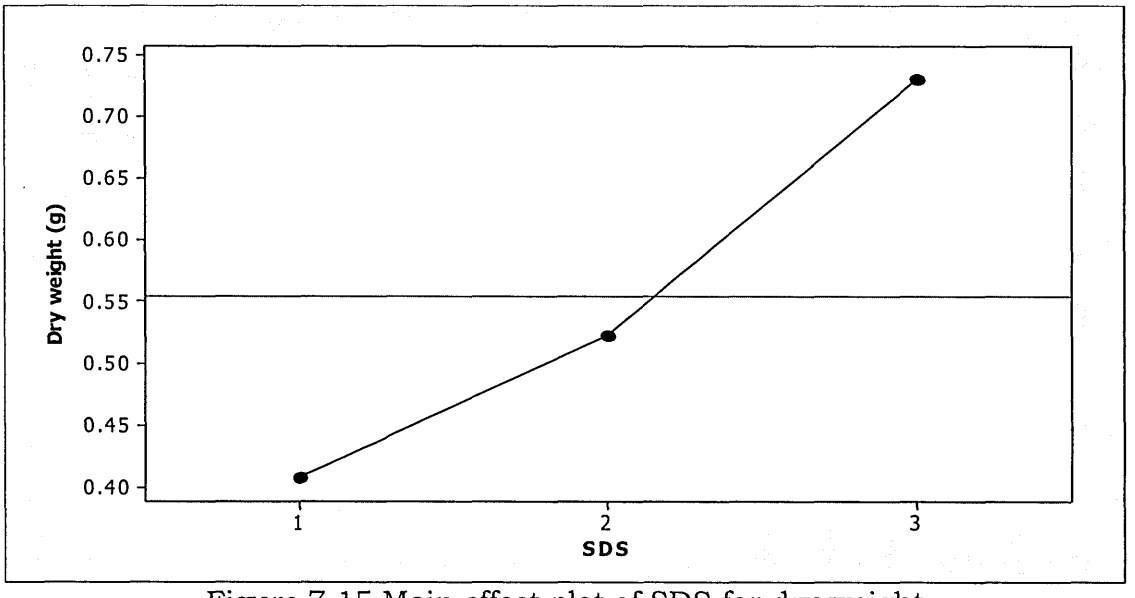


Figure 7.15 Main effect plot of SDS for dry weight

There was a perfect Spearman's rank correlation between observed means and SNR (r_s = 1.000) for dry weight. The five best treatment combinations selected for largest dry weight are summarised in Table 7.16.

Table 7.16 Five best treatment combinations selected for largest dry weight using the mean, their corresponding SNR_{Small} and ranks

SDS	BAP (g/l)	Mean (g)	rank	SNR	rank
3	1.5	0.736	1	-2.36	1
3	1.0	0.730	2	-2.41	2
3	0.5	0.728	3	-4.12	3
2	0.5	0.557	4	-5.19	4
2	1.5	0.533	5	-5.53	5

Note: All the treatment combination selected showed 4hr as optimal desiccation time

7.3.4.2.2 Overall post-cryopreservation germination

Analyses showed that for each response variable, only SDS had a significant effect (Table 7.17). Therefore, the overall viability as percentage germination is shown for this experiment, regardless of desiccation time and BAP level in the recovery media in Figure 7.16.

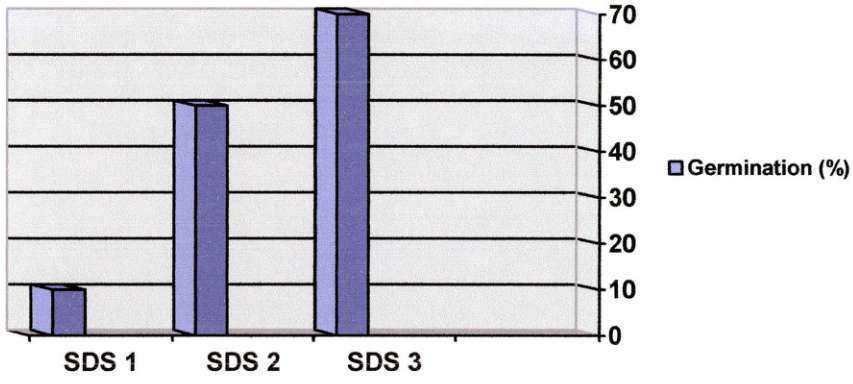


Figure 7.16 Percentage germination of *S. cordata* embryos after freezing in liquid nitrogen at three different maturity stages.

Table 7.17 Summary results for *S. cordata* cryopreservation experiment

Experimental Design	**Factorial full experiment**	**Taguchi L_{18} experiment**
Germination day		
Significant main effect		
SDS	S***	S***
Desiccation time (DT)	NS	NS
BAP	NS	NS
Significant Interactions	NS	NS
Spearman's rank correlation coefficient, r_s,		
Mean Vs. SNR	1.000	1.000
Shoot to root ratio		
Significant main effect		
SDS	S**	NS
Desiccation time (DT)	NS	NS
BAP	NS	S*
Significant Interactions		
SDS*BAP	NS	S**
Spearman's rank correlation coefficient, r_s,		
Mean Vs. SNR	0.783, p=0.013	0.783, p=0.013
Dry weight		
Significant main effect		
SDS	S***	S**
Desiccation time (DT)	NS	NS
BAP	NS	NS
Significant Interactions	NS	NS
Spearman's rank correlation coefficient, r_s,		
Mean Vs. SNR	1.000	1.000

*NS=Not significant, S=significant at p<0.05 for *, p<0.01 for ** and p<0.001 for ****

7.3.5 Discussion

Selecting an appropriate seed maturation stage is extremely important in the development of cryopreservation protocols especially for those seeds within an intermediate-recalcitrant category. Whilst orthodox seeds undertake maturation drying which programmes the switch from a developmental mode to a germinative mode (Kermode and Finch-Savage, 2002; Soeda *et al.*, 2005; Greggains *et al.*, 2000), many recalcitrant seeds especially in the tropics, do not undergo a maturation drying and therefore, do not experience reduced cellular metabolism and a clear physiological termination of seed development (Pammenter and Berjak, 1999). Furthermore, the physiological/developmental status of the seeds can change from and between collection batches and that for same species fruits bearing the seeds may hold seed of different developmental stages. As the cryopreservation studies move from orthodox to intermediate and recalcitrant seeds, their developmental and physiological characters become more complex and heterogeneous. Therefore, making the optimisation of cryopreservation protocol more difficult. Hence, the potential power and advantage of applying efficient experimental strategies was investigated in this chapter.

For many tropical tree seeds, development is followed by germination without interruption during fruit dehiscence and dispersal. It has been reported that those seeds (e.g. *Artocarpus heterophyllus* (Krishnapillay, 2000); *Swietenia macrophylla* (Marzalina and Normah, 2001); *Hopea odorata* (Nadarajan, 1999)), which do not undertake maturation drying, are usually large, with very well developed embryos. All these seeds increase in dry weight until fruit dehiscence, with a slight or no loss in fresh weight. However, a decreasing loss in water content is characteristic of several seeds that do not undertake maturation drying (Greggains *et al.*, 2000). For these, embryos growth may continue (increasing in dry weight) after dehiscence in the absence of enough water to promote germination. However, in the tropical forest, with very high rainfalls and humidity levels, the seeds shed from mother trees on humid soils and therefore continue hydrating and subsequently germinate.

7.3.5.1 Characterization of *Sterculia cordata* seeds

In this experiment, *S. cordata* seeds were studied at three different development stages, namely, SDS 3 (when the seeds are physiologically matured and shed from mother trees and competent to germinate), SDS 2 (seeds one week younger than SDS 3), SDS 1 (seeds two weeks younger than SDS 3). Table 7.8 showed that moisture content declined drastically as the seed matured from SDS 1 to SDS 2 but than stabilised at SDS 2 and no difference in moisture content was noted between SDS 2 and SDS 3. This observation was different from those reported for orthodox seeds where seed moisture content continues to decline until the seed is fully matured and ready to germinate (Bailly *et al.*, 2001).

In this study, it was noted that the seed dry weight continued to increase until the end of maturity (Table 7.8). During maturation, reserve material accumulation occurs to such a degree that dry weight increases in spite of the already initiated decrease in water content. Aberlence-Bertossi *et al.*, (2003), reported similar observation in oil palm (a recalcitrant seed producing species), where embryos underwent dehydration up to 120 days after pollination. But their moisture content remained high at maturity and their dry weight during maturation increased between 80 and 140 days after pollination and was then stable to the point of maturation.

In *S. cordata,* the percentage germination for non-cryopreserved embryos was relatively low (40%) for seeds harvested at SDS 1 (Table 7.8). However, it increased to 80% as the seeds matured to SDS 2 and to 100% at SDS 3. These data indicate that the seeds are naturally preparing for germination as they matured and optimum germination is attained at the end of maturation process.

7.3.5.2 Effects of maturation, desiccation and Benzyl amino purine (BAP)

Embryos at SDS 3 germinated most rapidly followed by those at SDS 2 and SDS 1. This observation supports the supposition that seeds at the SDS 3 are ready to germinate once placed in the appropriate medium, on which sprouting is triggered immediately. SDS 2 was noted to produce

seedlings with shoot to root ratios closer to one, as shown in the factorial analysis. However, Taguchi analysis demonstrated that shoot to root ratio was not affected by SDS. SDS was found to affect significantly dry weight of the seedling. Seedling showed greater dry weight at SDS 3 followed by SDS 2 and SDS 1. This observation explains that as the seeds get matured, they tend to have greater dry matter content compared to immature seeds and those seeds with greater dry weight might have produced seedlings with greater dry weights. This may explain why all the best five treatments selected for greater dry weight have SDS 3 (Table 7.12)

The percentage germination before cryopreservation showed that SDS 1 embryos are extremely sensitive to desiccation and their germination cannot therefore be improved by desiccation. All embryos were dead after 6hr desiccation. Percentage germination increased when seed moisture content declined naturally as the seeds matured (Table 7.8). However, when this outcome was compared to experimentally-induced desiccation, germination of *S. cordata* embryos in all the SDSs was not improved. It was reported that bean (*Phaseolus vulgaris*) seed tolerance to drying developed fully only at the end of seed maturation, approximately 50 days after anthesis (Bailly *et al.*, 2001). Bailly *et al.*, (ibid) also confirmed that drying of very young seeds, which were not at all tolerant to desiccation, induced strong deterioration of cell membranes as indicated by high electrolyte leakage. In this study, embryos from all SDSs also showed a decline in germination as they were desiccated for longer periods. The same observation was also reported for the rapid drying of Norway maple (*Acer platanoides*) seeds collected 21 days before mass maturity (Hong *et al.*, 2000). However, Nedeva and Nikilova (1999), reported an increased germination with desiccated premature *Triticum aestivum* (wheat) seeds.

7.3.5.3 Post-cryopreservation recovery

In this study, freezing tolerance in developing *S. cordata* seeds was identified by their embryos' ability to germinate after immersion in liquid nitrogen. The data in the present study showed that post-cryopreservation

germination of developing seeds increased abruptly as the seeds mature and when their moisture content was lower.

The embryos survived liquid nitrogen storage with a percentage germination of 70% at SDS 3 and about 40% at SDS 2 followed by 10% at SDS 1. SDS 3 was found to be optimum for cryopreservation of the *S. cordata* embryos. The reduced germination at SDS 2 and SDS 1 indicated that tissues were damaged at water contents much higher than those of seeds at SDS 3. The percentage germination continued to increase with seed maturation as the moisture content decrease and dry weight increase. Increase in post-cryopreservation germination may also be due to other seed physiology factors which may have reduced freezable water content, thereby, allowing seed survival.

During maturation, when the seeds have initiated the desiccation process, some solutes were evidently still accumulating, as demonstrated by the increase in dry weight (Table 7.8). These results may indicate that the reduction in seed water content may not completely explain the greater acquisition of freezing tolerance for embryos at SDS 3 as compared to those at SDS 2. It was observed that freezing tolerance increased sharply at the later stage of seed development when there is an accumulation in dry weight. This phenomenon may be related to the increase of the so-called late embryogenesis accumulated (LEA) proteins, which are involved in the maturation and desiccation tolerance acquisition (Grelet *et al.*, 2005; Tabaei-Aghdaei *et al.*, 2003), as well as other solutes such as oligosaccharides (Hong *et al.*, 2000; Bentsink *et al.*, 2000). Oligosaccharides also reported to have role in seed longevity especially in achieving intracellular glass stability during freezing Buitink *et al.*, (2000). The raffinose family of oligosaccharides has been reported as endogenous cryoprotectants, which accumulate during the cold hardening of Puma rye (Koster and Lynch, 1992). Moreover, raffinose is a crucial oligosaccharide for the prevention of sucrose crystallization in drying embryo axis, promoting glass formation, a phenomenon which is prerequisite for the survival of tissues to freezing at ultra-low temperatures (Koster, 1991).

7.3.5.4 Comparisons of experimental designs

For both experiments with and without LN storage, both factorial and Taguchi full experimental analyses showed that similar main effects were significant for each of the three response variables (Tables 7.13 and 7.17). However, the Taguchi experiment showed a slightly different result from their factorial counterparts when identifying two-way interactions. Muthusamy *et al.,* (2005) showed that same conclusions as factorial experiments could be obtained with Taguchi experiments in two level factorial experiments. Furthermore, they claimed that more consistent conclusions are obtained using the Taguchi methodology.

For the experiment without LN storage, comparisons of full experiments with $^1/_3$ fractionally replicated experiments, also showed almost similar results for both methodologies used (ANOVA and SNR). However, no two-way interaction could be investigated in the one-third replicated experiment. The full factorial experiment showed that there are many significant two-way interactions especially for shoot to root ratio and dry weight assessments (Table 7.13). Therefore, if only the one-third factorial experiment was to be carried out the information on two-way interaction will be lost. However, this one-third fractional experiment is useful if only main effects are to be investigated or to carry out screening or preliminary experiments or when there is limited number of samples to run a big experiment (Babiak *et al.*, 2000). For the (2×3^2) experiment with LN storage, no smaller fractional replication is possible. However, if another level is added for desiccation time factor to produce a 3^3 experiment (similar to experiment without LN storage) then a one-third replication of the original experiment could be used to investigate all the main effects.

7.3.6 Conclusions

1. A successful cryopreservation method has been developed for *S. cordata* zygotic embryos using desiccation.

2. From both the factorial and Taguchi experiments, SDS and desiccation time were identified as the most important factors in the experiment without liquid nitrogen storage. Germination increased with maturation but decreased with desiccation time.

3. Germination after cryopreservation was only affected by seed development stage, where embryos at SDS 3 showed greater germination.

4. The capacity of desiccated, immature embryos (SDS 1 and SDS 2) to germinate does not depend on the level of desiccation achieved by artificial drying, but only on their developmental stage at the time of harvest.

5. The Taguchi experiment was successfully applied to the cryopreservation *S. cordata* embryos, providing similar conclusions as the factorial experiment.

6. Comparisons of full experiments with the one-third fractionally replicated experiments, also showed similar results for both statistical methodologies (ANOVA and SNR) used.

7.3.7 Further Progress

Having identified the optimal developmental stage for cryopreservation of *S. cordata* embryos, another method of cryopreservation using vitrification was investigated for this species in the next section.

7.4 Cryopreservation of *Sterculia cordata* Zygotic Embryos Using Vitrification

7.4.1 Introduction

Cryopreservation of tropical forest tree germplasm is one of the important *ex situ* conservation methods applied at the Forest Research Institute of Malaysia (FRIM). It is used to preserve not only in base genebanks, but also in 'working' active germplasm repositories that support biotechnologically-based initiatives including micropropagation and tissue culture which support tree improvement and development programmes (Krishnapillay, 2000). Therefore, the exploration of different cryo-conservation methods that present different storage stresses, tolerances and cryoprotective modalities is important in expanding the cryopreservation protocol options available for conserving physiologically and genotypically diverse tropical tree seeds. The previous study investigated cryopreservation of *S. cordata*, an intermediate seed producing species using desiccation technique (Section 7.3). This study explores the possibility of using vitrification technique for cryopreservation of this species.

The process of vitrification is now becoming one of the main approaches in the cryopreservation of plant germplasm for long-term conservation (Engelmann, 2004). Vitrification refers to the physical process by which a concentrated aqueous solution solidifies into stable amorphous glass at a sufficiently low temperature without ice crystallization (Taylor *et al.*, 2004). Complete vitrification eliminates the potentially damaging effect of intra- and extra cellular crystallization and produces high levels of cell survival (Benson, 2004).

In this experiment, cryopreservation of *S. cordata* zygotic embryos using PVS2 was explored for the first time. This procedure is known as two step vitrification, where it involves dehydrating germplasm by a loading solution (LS) containing 2M glycerol and 0.4M sucrose at 25°C, followed by exposure to highly concentrated vitrification solution (PVS2) and vitrified samples transferred directly into LN (Matsumoto and Sakai, 2003).

Only seeds at developmental stage three were used for this experiment since the results of the previous experiment on this species showed that developmental stage three had greater germination potential after cryopreservation. The whole experiment was carried out in three phases. The first phase consists of a sucrose preculture treatment, the second comprises adding a loading solution (LS) treatment and the third includes the PVS2 treatment. The potential toxicity effect of each cryoprotective treatment was assessed by conducting germination test at each phase before the zygotic embryos were introduced to the next successive phase of cryoprotective treatment.

7.4.2 Materials and methods

7.4.2.1 Seed material

Seed collection, surface sterilization and embryo excision were carried out as described in Section 7.3.3.

7.4.2.2 Experimental procedures

Phase I. sucrose preculture treatment: The excised embryos were precultured for three days on solidified MS medium (Murashige and Skoog, 1962), supplemented with three different 0.35, 0.55 or 0.75 M sucrose treatments. At the end of these treatments, 10 embryos were selected for moisture content testing, 10 for toxicity testing and 10 more for cryostorage for each treatment.

Phase II. treatment with LS: The effects of increasing exposure time to the loading solution were evaluated for the embryos after preculture in sucrose. Loading solution (comprising 0.4 M sucrose and 2 M glycerol) was applied to the embryos for one, two and three hours at 25°C. Ten embryos were used for the toxicity trial and a further 10 embryos were tested for germination after cryopreservation for each treatment combination.

Phase III. treatment with PVS2: After the sucrose preculture and LS treatments, the embryos were then treated with PVS2 [30% (w/v) glycerol, 15% (w/v) ethylene glycol, 15% (w/v) DMSO and 0.4 M sucrose] for one, two and three hours at 0°C (Sakai, 2004). After these treatments, the vitrification solution was replaced by 0.5 ml liquid MS medium with 1.2 M sucrose for 20 minutes. The embryos were then blotted dry on sterile filter paper. The effect of the exposure time of PVS2 solution on germination of the embryos was investigated before and after cryopreservation.

7.4.2.3 Cryopreservation and recovery

Cryopreservation was carried out by rapid cooling into liquid nitrogen (+LN) for each treatment. After 24 hours of storage in LN, placing them in a water bath at 38 + 2°C for 10 to 15 minutes rapidly rewarmed the embryos. Germination (radicle emergence) was assessed before and after cryopreservation for each step. The embryos were placed on standard semi-solid medium with 0.3 M sucrose followed by standard medium with 0.1 M sucrose for growth recovery and incubated in a temperature-controlled growth room at 25 $\pm$ 1°C with a 12 hours photoperiod and 60 $\mu mol.m^{-2}.s^{-1}$ light intensity. The development of each embryo was observed daily for four weeks. Germination was defined as embryos that swelled and elongated and eventually grew into complete plantlets (Figure 7.17).

7.4.2.4 Data analysis

Data analysis was by binary logistic regression (Refer Section A2.2.3) as the primary response was the number of germinated embryos from ten replicates, which follows a binomial distribution.

7.4.3 Results

The initial moisture content of the embryos was approximately 26%. It declined to 24, 21 and 19% after three days of preculture on MS media supplemented with 0.35, 0.55 and 0.75 M sucrose respectively.

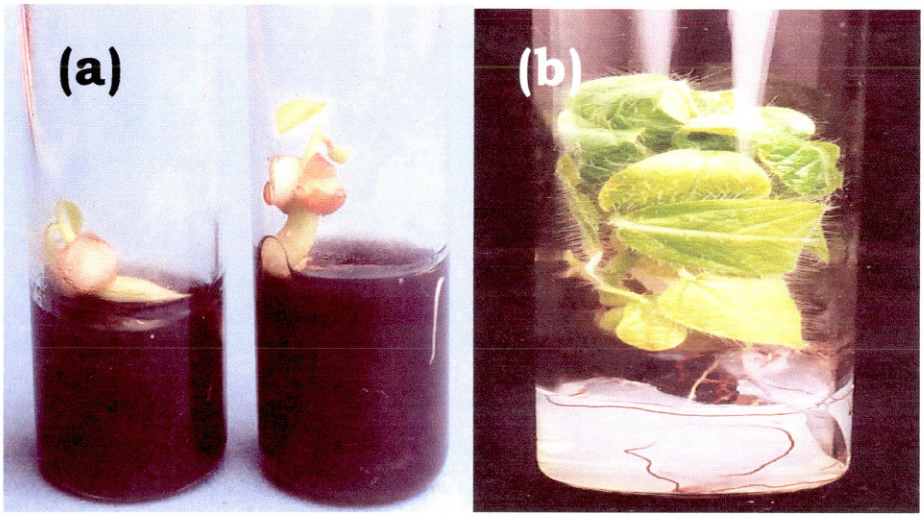


Figure 7.17. *Sterculia cordata* embryos growing in recovery medium after two weeks (a) and four weeks (b) of germination

7.4.3.1 Phase I: sucrose preculture treatment

The main effects plot showing the number of germinated embryos (from the total of 10) before cryopreservation suggests a possible quadratic (non linear) relationship with sucrose concentration (Figure 7.18). However, binary logistic regression analysis showed that fitting sucrose and its quadratic is not significant [G^*=0.401, DF=2; p>0.05] (Model 2; Table 7.18). This analysis also showed that adding LN to the model (Model 3; Table 7.18) significantly improves the model predicting embryo germination (G^*=69.773, DF=1; p<0.001). This suggests that all the levels of sucrose produce similar germination rate before cryopreservation. Since germination above 90% was achieved (Figure 7.19a), sucrose does not have detrimental effect on the embryo germination before cryopreservation. However, there is a significant difference on the germination of embryos before and after cryopreservation. Figure 7.19a shows the fitted values for percentage germination for sucrose preculture treatment (regardless of their levels) before and after

cryopreservation. The fitted value for percentage germination declined from above 90% before cryopreservation to about 3.0 % after cryopreservation for this pre-treatment showing that this treatment alone is not sufficient to achieve high germination after cryopreservation. Therefore, the LS treatment was added after this pre-treatment.

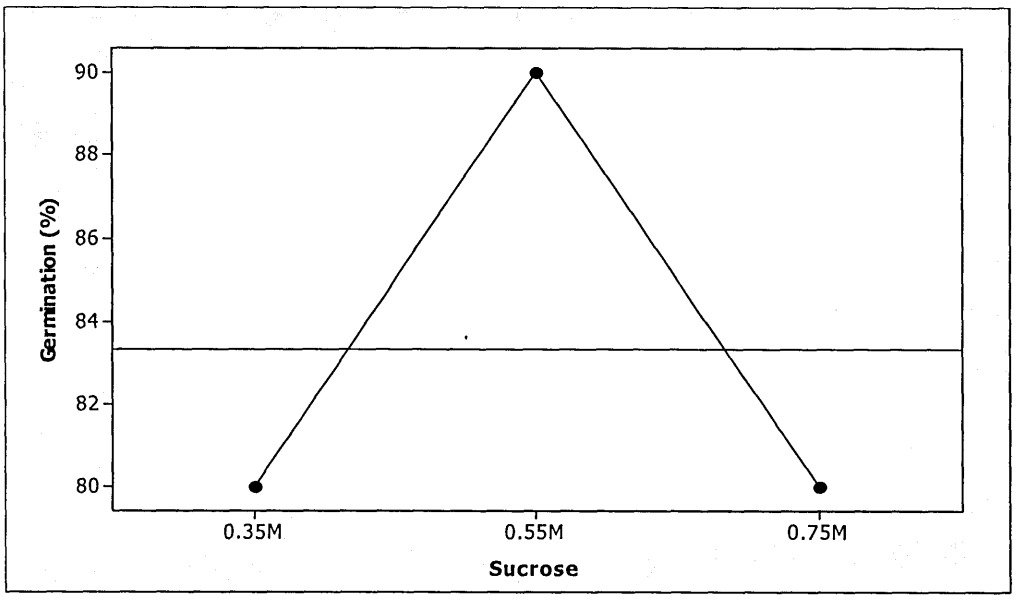


Figure 7.18 Main effects plot for number of germinated embryos before (-LN) for sucrose preculture treatment.

Table 7.18 Analysis of deviance for sucrose pre-growth treatment

Model	Deviance explained, *G*	*DF*	Deviance difference, *G**	*DF* difference	*P*
1.Null	0	0			
2.Sucrose+sucrose^2	0.401	2	0.401	2	>0.05
3.Sucrose+sucrose^2+LN	70.174	3	69.773	1	<0.001

DF = degrees of freedom

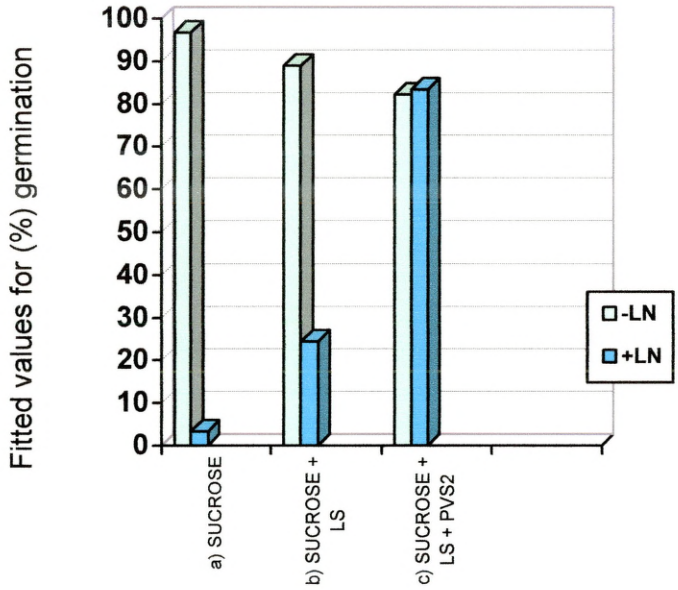


Figure 7.19 Fitted values for germination of *S. cordata* embryos after sucrose, LS and PVS2 treatments (regardless of their levels) before (-LN) and after cryopreservation (+LN).

7.4.3.2 Phase II: treatment with loading solution (LS)

Main effect plots also indicated a quadratic relationship with LS treatment for embryo germination (Figure 7.20).

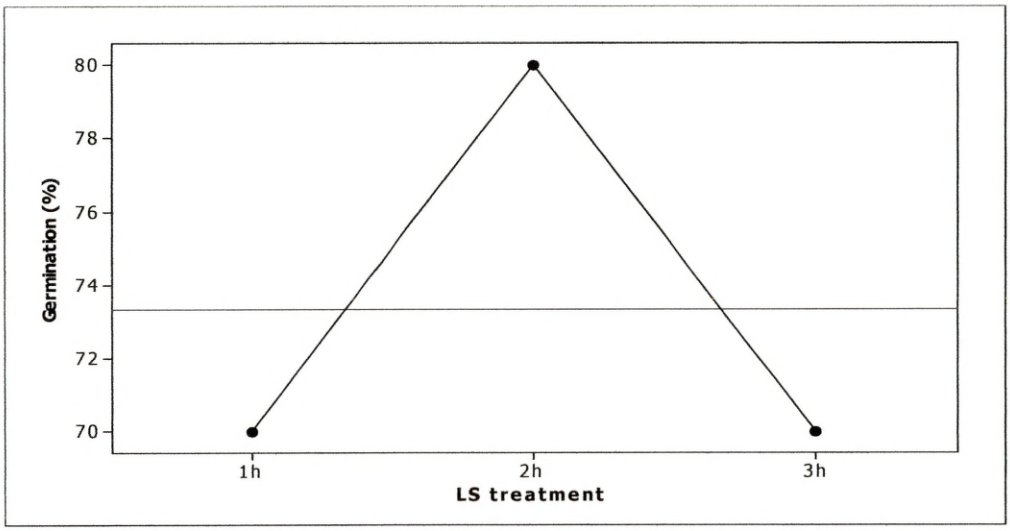


Figure 7.20 Main effects plot for number of germinated embryos before (-LN) for sucrose pre-treatment.

However, binary logistic regression analysis showed that fitting the full quadratic model involving sucrose, LS and their interaction does not have a significant effect on the germination of the embryos before cryopreservation [G^*=4.848, DF=5; p>0.05] (Model 2; Table 7.19). This indicates that LS treatment for one, two and three hours have same effect and since the percentage germination was above 80% for all these treatments, it shows that sucrose pretreatment was not lethal to *S. cordata* embryos. However, adding LN to the model (Model 3; Table 7.19) significantly improved the model predicting embryo germination (G^*=87.146, DF=1; p<0.001) suggesting that there is a significant different in germination of the embryos before and after cryopreservation. Germination before cryopreservation was about 85% and it declined to about 20% for cryopreserved embryos after sucrose and LS treatments (Figure 7.19b). This result suggests that though there is a slight increase in germination after sucrose and LS treatment, they are not sufficient to achieve high germination after cryopreservation. This would be an expected result from a cryopreservation context however, this step was included as a progression through a logical deductive process to substantiate the experimental design. In the next phase of this experiment, PVS2 treatment was added.

Table 7.19 Analysis of deviance for sucrose and LS treatments

Model	Deviance explained	*DF*	Deviance difference, G^*	*DF* difference	*P* value
1.Null	0	0			
2.Sucrose+LS+sucrose*LS+sucrose^2+LS^2	4.848	5	4.848	5	>0.05
3.Sucrose+LS+sucrose*LS+sucrose^2+LS^2+LN	91.994	6	87.146	1	<0.001

DF = degrees of freedom

7.4.3.3 Phase III: treatment with PVS2

The main effects plot for germination of the embryos also indicated that there was a possible quadratic relationship for the PVS2 treatment (Figure 7.21).

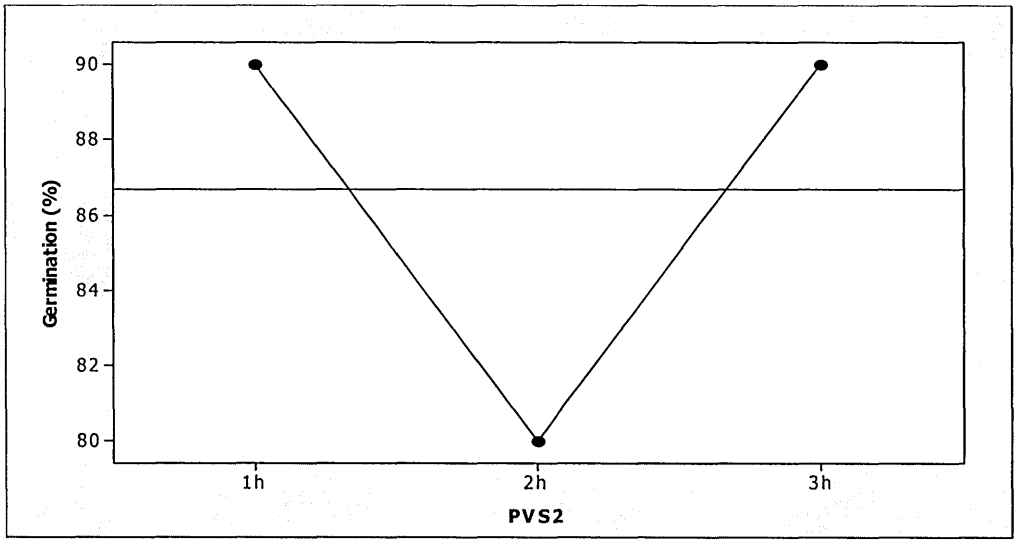


Figure 7.21 Main effects plot for number of germinated embryos before (-LN) for sucrose pretreatment.

The binary logistic regression analysis showed that fitting the full quadratic model involving sucrose, LS and PVS2 treatment and their interactions does not have a significant effect on the embryo germination before cryopreservation [G^*=1.972, DF=9; p>0.05] (Model 2; Table 7.20). High germination above 80% before cryopreservation was achieved after PVS2 treatments regardless of the duration of treatment (Figure 7.19c). Adding LN to the model does not significantly affect the model predicting the embryo germination [G^*=0.117, DF=1; p>0.05] (Model 3; Table 7.20) suggesting that there was no significant difference between germination of cryopreserved and non-cryopreserved embryos. High germination (>80%) was also achieved after PVS2 treatment for cryopreserved embryos (Figure 7.19c). These results suggested that PVS2 was not only non-toxic to the embryos but that it also acts as an excellent cryoprotectant. Since greater post-cryopreservation germination, which was almost similar to the control was achieved after this treatment (Figure 7.19c).

Table 7.20 Analysis of deviance for sucrose, LS and PVS2 treatments

Model	Deviance explained	*DF*	Deviance difference, *G**	*DF* difference	*P* value
1. Null	0	0			
2. Sucrose+sucrose^2+LS+LS^2+PVS2+PVS2^2+ Sucrose*LS+Sucrose*PVS2+LS*PVS2	1.972	9	1.972	9	>0.05
3. Sucrose+sucrose^2+LS+LS^2+PVS2+PVS2^2+ Sucrose*LS+Sucrose*PVS2+LS*PVS2+LN	2.089	10	0.117	1	>0.05

DF = degrees of freedom

7.4.4 Discussion

A successful cryopreservation protocol was developed for *S. cordata* zygotic embryos using a vitrification method for the first time in this experiment. A requirement for successful cryopreservation is often the preculture phase involving the culture of plant material on a medium containing cryoprotectants (generally sugars such as sucrose or glucose) followed by the rapid cooling of the explants in LN. A high level of sugar accumulated during preculture has been reported to improve survival of cryopreserved materials (Kamata and Uemura, 2004). Sucrose partially dehydrates samples through osmotic effects and it is also absorbed by plant samples, thereby increasing the concentration of intracellular solutes and hence, increases the tolerance to dehydration (Dumet *et al.*, 2000a). The decrease in moisture content (from 24, 21 and 19% respectively) in the embryos of *S. cordata* after preculture in 0.35, 0.55 and 0.75 M sucrose confirmed the dehydration effect of sucrose. High germination was obtained for these embryos after all sucrose preculture treatments regardless of the concentration of sucrose before cryopreservation (Figure 7.19a) indicating these high levels of sucrose (0.35, 0.55 and 0.75 M) are not detrimental to *S. cordata* embryos. Similar observations were reported for *Citrus aurantifolia* embryonic axes (Cho *et al.*, 2002). However, preculture of *S. cordata* embryos with sucrose alone did not lead to a substantial increase in the survival (~3%) after cryopreservation (Figure 7.19a).

To improve the germination rate, the effects of LS were examined on the embryos. LS treatment for one, two and three hours (after pre-growth in

different levels of sucrose) was also not detrimental to the embryos. However, the percentage germination after cryopreservation for this pre-treatment was still low (about 20%). The increase in germination after cryopreservation from about 3% with sucrose preculture alone to about 20% after LS treatment shows that this additional pretreatment increases the germination compared to treatment with sucrose alone but it does not give a satisfactorily high germination after cryopreservation.

According to Matsumoto and Sakai (2003), during LS treatment, cells were osmotically dehydrated and considerably plasmolyzed and when these plasmolyzed cells were subsequently subjected to PVS2 solution, the cells successively decreased in cytosolic volume and remain osmotically dehydrated. The protective effect of LS solution after preculture might be caused by osmotic dehydration, resulting in the concentration of cytosolic stress-responsive solutes accumulated during preculture with high concentration of sucrose and in the protective effects of plasmolysis (Sakai, 2004). The presence of a highly concentrated cryoprotective solution in the periprotoplasmic space of plasmolyzed cells may mitigate the mechanical stress caused by successively severe dehydration (Jitsuyama *et al.*, 1997). These intracellular and extracellular protective effects may minimize the injurious membrane changes during severe dehydration. Thus the LS treatment following preculture with sucrose enriched medium is a very promising step for successful cryopreservation by vitrification.

To improve germination after cryopreservation, *S. cordata* embryos were treated with PVS2 following preculture in high sucrose medium and LS treatment. PVS2 treatment did not significantly affect the germination of the embryos before cryopreservation. For many species, highly concentrated vitrification solutions were reported to be harmful (Abdelnour-Esquivel and Engelmann, 2002), however, higher survival was reported for a number of species after vitrification (Matsumoto and Sakai, 2003; Sakai, 2000). This treatment had a significant effect on the germination of *S. cordata* embryos after cryopreservation. In Figure 7.19c, the fitted value for germination increased from 20% without PVS2 treatment to above 80% with PVS2 after cryopreservation regardless of the duration of treatment.

The vitrification-based approach is more suitable for complex organs, such as shoot-tips and embryos that contain a variety of cell types, each with unique requirements under conditions of freeze-induced dehydration (Engelmann, 2004). By precluding ice formation in the cell system, vitrification-based procedures are operationally less complex compared to traditional controlled rate freezing methods and have a greater potential for broad applicability.

The application of an appropriate experimental design and data analysis is very important in any cryobiological investigation (Motulsky, 1995; Muthusamy *et al.*, 2005). An inappropriate analysis could lead to misleading conclusions. In this experiment though the main effect plots suggested that there were quadratic effects of sucrose preculture, LS and PVS2 treatments, the binary logistic regression analysis showed that the quadratic effects were not significant. This analysis is suitable for this experiment since there were low numbers of replicates and the data were a binomial distribution (Sokal and Rohlf, 1995).

With high recovery rates after freezing for *S. cordata* zygotic embryos, vitrification is a reliable, reproducible cryogenic technique for cryopreserving valuable tropical intermediate-recalcitrant seed producing species which are more difficult to store using conventional seed conservation methods.

7.4.5 Conclusions

1. A vitrification-based cryopreservation protocol has been developed for *S. cordata* zygotic embryos.

2. Sucrose concentration in preculture media, LS and PVS2 pre-treatments are not detrimental to the embryos of *S. cordata.*

3. Sucrose preculture treatment alone and together with LS treatments are not sufficient to achieve high germination after cryopreservation.

4. Additional PVS2 treatment is necessary to obtain high germination (≥ 80%) after cryopreservation for *S. cordata* embryos.

5. The correct conclusions from these experiments were assisted by use of appropriate data analysis (binary logistic regression analysis).

7.4.6 Summary

This chapter concludes three cryopreservation experiments on two tropical intermediate species. As the species recalcitrance increases it was more difficult to develop cryopreservation protocol. The seed physiology plays important role in successful cryopreservation especially for non-orthodox seeds. Therefore, this factor was considered for cryopreservation protocol development in this chapter, which was not included in the previous chapter. The next chapter will focus on developing a cryopreservation protocol by taking a different approach from the previous studies of orthodox and intermediate seeds for a recalcitrant tropical seeds, which has proven difficult to cryopreserve.

CHAPTER 8

CRYOPRESERVATION OF SHOOT-TIPS OF *PARKIA SPECIOSA*, A RECALCITRANT SEED PRODUCING SPECIES USING ENCAPSULATION-VITRIFICATION

8.1 Introduction

The studies in this thesis have so far considered orthodox and intermediate seed and embryo storage by cryopreservation. Each of these categories has some level of tolerance to cryopreservation. This is not the case for recalcitrant seeds for which there are many issues related to storage problems. These include intolerance to cryogenic parameters, high metabolic activity and also seed availability. Therefore, the study in this chapter takes a different approach to investigate the development of rationales and procedures for recalcitrant seed germplasm. These are based on:-

1. Using shoot-tips from germinated seeds and their *in vitro* derived micropropagated plantlets as an approach to overcome seed limitation problem.
2. The application of Differential Scanning Calorimetry to elucidate the critical points at which vitrification occurs in the shoot-tips such that cryoprotection is achieved.
3. The novel application of trehalose cryoprotectant (which has been highly related to survival of polar biota as alternative to sucrose) in the preculture medium.

These exploratory approaches will be considered as this chapter progresses in the context of future applications incorporating novel statistical procedures.

8.2 Species Introduction

The cryopreservation studies in this chapter were carried out on *Parkia speciosa* a recalcitrant seed producing species. *P. speciosa* (locally known as petai in Malaysia) belongs to the family of Leguminosae. It is native to Southeast Asia and widely distributed from Southern Thailand, Peninsular Malaysia, Sumatra, Borneo and the Philippines (Sosef *et al.*, 1998). The tree is medium-sized to large, growing up to 50m tall with the columnar bole having an average 100-250cm diameter (Figure 8.1a). *Parkia speciosa* occurs scattered in lowland rainforest, on sandy, loamy and

podzolic soils, also in waterlogged locations, in fresh water swamp forest and on riverbanks (Whitmore, 1972).

P. speciosa species is not grown commercially, but is found in small plantings around villages and is relatively common in native forests (Lensen, 1995). Their seedlings are collected by farmers from the wild and planted in their home garden or fields to use as a vegetable and medicinal plant. The light-bulb shaped inflorescences of these trees are suspended from pendulous stalks, permitting easy access to bats and other pollinators (Sosef, 1995). *P. speciosa* fruit is a leathery or woody, stalked, oblonged large pod (35-55 cm long and 3-5 cm wide), straight or more commonly twisted; dangling in small green bundles and usually indehiscent. Each pod contains 10-18 large (2cm width and 3-5cm diameter; Figure 8.1b) seeds. The seeds in the pods are usually in one row, ellipsoid, with fleshy cotyledons. Hornbills, monkeys, squirrels, deer, elephants and wild pigs feed on the fruits and leading to seed dispersal (Lensen, 1995).

P. speciosa seeds are commonly used as a vegetable; they are prized for their garlicky taste and are used to flavour curries and other traditional dishes (Weng and Lim, 1994). Young leaves and the receptacles of the inflorescence are occasionally eaten. Seeds of *P. speciosa* contain the essential amino acid cystine. The seeds are known to be hypoglycemic (reducing blood sugar level) and are used traditionally for treating kidney pain, cancer, diabetes, hepatalgia, oedema, nephritis, colic, cholera and as an anthelmintic; they are also applied externally to wounds and ulcers. The Forest Research Institute of Malaysia (FRIM) estimates the local value of petai at over a million dollars annually in Peninsular Malaysia, not including its potential value as a timber tree, a use which is now being tried experimentally (Weng and Lim, 1994).

Figure 8.1. *Parkia speciosa* tree (a), seedpods (b) and seeds (c).

8.3 Cryopreservation of Recalcitrant Seeds

P. speciosa seeds are shed from their mother trees with a very high (around 80%) moisture content. This species exhibits a typical recalcitrant seed storage behaviour as the seeds are extremely sensitive to desiccation and low temperature. Therefore, long term storage of this species using conventional methods is impossible. Cryopreservation was suggested for long-term storage of many recalcitrant species (Engelmann, 2004). In this thesis, therefore, cryopreservation of tropical recalcitrant germplasm is extremely challenging since cryopreservation methods need to be applied to species that have never experience cold or desiccation, much less freezing in their normal life cycle. The development of a cryopreservation protocol for these recalcitrant seeds needs to include both cryogenic (cryoprotectant and

low temperature treatments) and non-cryogenic (pre-and post-storage culture) components taking into consideration parameters such as the physiological status of the plant germplasm, desiccation and freezing sensitivity of the material and recovery medium (Reed *et al.*, 2005). Furthermore, recalcitrant seeds are often bulky with fleshy cotyledons, metabolically active and highly hydrated which can make it difficult to cryopreserve the whole seed.

Some of the earlier studies, embryo cryopreservation of tropical species were carried out on *Cocos nucifera* (Bajaj, 1985; Chin *et al.* 1989), oil palm (Grout *et al.*, 1983), *Howea* and *Veitchia* palms (Chin *et al.*, 1988), *Hevea* (Normah, 1987), and rambutan (Chin *et al.*, 1988). Embryos of recalcitrant seeds of forest tree germplasm were cryopreserved at the Seed Technology Section at FRIM. Chai *et al.*, (1994) reported the cryopreservation of *Shorea leprosula, S. ovalis, S. parvifolia, S. macrophylla* and *Hopea odorata* embryos. However, the recovery of the embryos was low (between 5-15 %) and the viable seedlings were reported to grow very slowly. Other serious problems reported were the formation of abnormal seedlings and microbial contamination. Successful cryopreservation of shoot-tips or apices of tropical recalcitrant seed producing species have also been reported in banana (Agrawal *et al.*, 2004), Cocoa (Jong-Yi *et al.*, 2004), chayote (Abdelnour-Esquivel and Engelamnn, 2002) and many other species (Engelmann, 2004).

In this study, the shoot-tips of *P. speciosa* were cryopreserved for the first time using the encapsulation-vitrification method. Trehalose was used in the preculture medium as an alternative to commonly used sucrose. The widespread presence of trehalose in polar biota is related to acclimatisation to both low temperature and partial dehydration (Montiel, 2000). This study investigated if tropical recalcitrant species could adopt the survival mechanism of polar biota in the extreme cold weather. Thermal analysis was carried out using DSC to investigate the effect of cryoprotective treatments in eliminating osmotically active water and achieving a glass transition phase during cryopreservation.

8.4 Materials and Methods

8.4.1 Seed material

Seeds were collected from mother trees near the FRIM campus. The seeds were extracted from the seedpods and then washed in running water, surface sterilized (10% v/v hypochlorite solution, Domestos, Lever Bros.) for 10 minutes and with 0.3% (w/v) boric acid for 1 minute, followed by rinsing them once in 30% (v/v) ethanol for 1 minute and three times in sterile distilled water.

8.4.2 *In vitro* seed germination

Surface sterilized seeds were cultured on Murashige and Skoog (1962) medium (MS) supplemented with 30g/l sucrose (Duchefa Biochemie) and 5.5g/l Plant Agar (Duchefa Biochemie) and incubated in a temperature-controlled growth room at 26°C with a 12 hours (60 $\mu mol.m^{-2}.s^{-1}$) photoperiod.

8.4.3 Preculture on trehalose medium

After four weeks of germination, shoot-tips (0.8-1.0 cm) were excised from the *in vitro* seedlings aseptically and cultured in Petri dishes containing Murashige and Skoog (MS) medium with three different concentrations of trehalose [2.5, 5 and 10% (w/v)]. The shoot-tips were precultured for one, two and three days in a temperature-controlled growth room at 26°C with a 12 hours (60 $\mu mol.m^{-2}.s^{-1}$) photoperiod.

8.4.4 Encapsulation of shoot-tips

At the end of preculture period, the shoot tips (5 mm) were excised from the pre-cultured shoots, encapsulated in alginate beads (low viscosity, 3% [w/v] Sigma Chemical) prepared with 0.4M sucrose MS medium free of calcium salts) and allowed to polymerise for 20 min in a 0.1M $CaCl_2$ solution at 26°C.

8.4.5 Treatment with PVS2

The encapsulated shoot-tips were dehydrated with PVS2 [30% (w/v) glycerol, 15% (w/v) ethylene glycol, 15% (w/v) DMSO and 0.4M sucrose] for 30, 60 and 90 min at 0°C in a 1.2 ml Nalgene plastic cryotubes (five bead/ cryotubes) (Sakai, 2004).

8.4.6 LN storage

After dehydration, the beads containing shoot-tips were then transferred to cryotubes containing one ml of fresh PVS2 solution and plunged into liquid nitrogen. These samples were then rapidly warmed for 20 min at the temperature of 40°C. After these treatments the shoot-tips were rehydrated for 20 min in liquid MS medium with 1.2M sucrose.

8.4.7 Post-cryopreservation recovery

The shoot-tips were plated on semi-solid MS medium for 24 hr followed by solid MS medium. These cultures were kept in the dark at 18°C in a growth cabinet for seven days with serial subculture once every two days. After seven days, the cultures were placed in a growth room at 26°C with a 12 hours (60 $\mu mol.m^{-2}.s^{-1}$) photoperiod.

8.4.8 Differential Scanning Calorimetry (DSC) thermal analysis

Thermal analysis during cooling and warming of encapsulated petai shoot-tips was undertaken using Differential Scanning Calorimetry (DSC) model Perkin Elmer DSC with Pyris seven software. The instrument was calibrated with zinc and indium and pure water as a standard for cryogenic operations (Benson *et al.*, 2004). Individual sample beads, were placed in aluminium pans, sealed with the aid of a Perkin Elmer crimper and weighed to record fresh weight values. Scans were performed from +25°C to –150°C with a scanning rate of $\pm$ 10°C min^{-1} for control and treated shoot-tips (modified from Benson *et al.*, 1996b). The treatments used for DSC are shown in Table 8.1. The shoot-tips were precultured for three days in trehalose for all the treatments selected. The purpose of DSC thermal analysis in this study is to determine phase transitions and associated thermo-physical properties for the cryopreserved *P. speciosa* shoot-tips at

every cryoprotective treatment to investigate the efficiency of the treatment in eliminating the freezable water and achieve glass transition phase during cryopreservation.

8.4.9 Measurement of water content

After thermal analysis, the lids of the sample pans were pierced and the pan together with the sample were dried in air at 100°C for a minimum of 24 h to determine the sample dry weight. This allowed the calculation of the total water content of a bead with and without a shoot-tip. Thermodynamically, as 1g of water releases 334.5 joules of heat energy when converted into ice and vice versa (Block, 2003), the osmotically active water content of the sample (g of osmotically active water per g of dry weight) was calculated from the endothermic–heat changes derived from the melt endotherm during the warming cycle along with the total water content of the sample. Osmotically inactive water is calculated as the difference between total water and osmotically active water contents. The water contents of fresh, untreated (control) and treated shoot tips were measured in the same way. The experimental procedures are illustrated in Figure 8.2.

Table 8.1 Treatments selected for thermal analysis using DSC for *P. speciosa* shoot-tips

Pre-growth in Trehalose (%)	**PVS2 Treatment (min)**
control	control
2.5	30
2.5	60
2.5	90
5.0	30
5.0	60
5.0	90
10	30
10	60
10	90

*Two replicates were used for each treatment. The control shoot-tips were not preculture in trehalose medium and not treated with PVS2

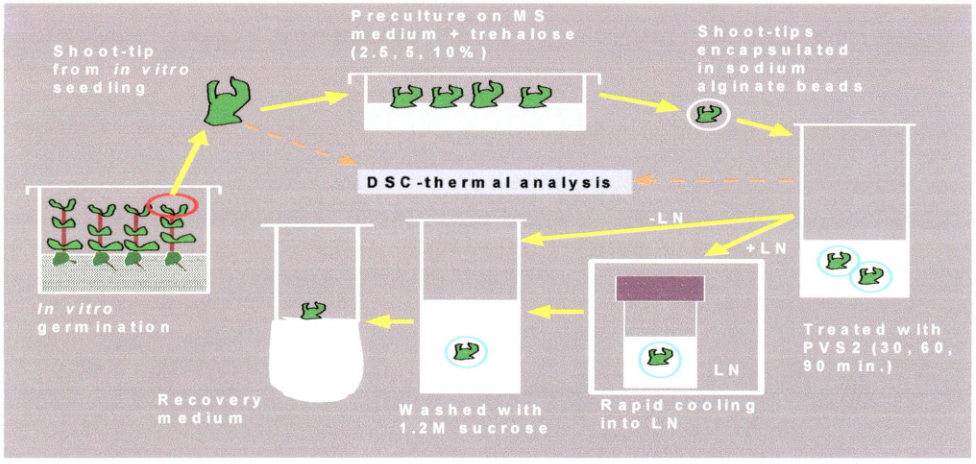


Figure 8.2 Experimental procedures for cryopreservation *P. speciosa* incorporating micropropagation and thermal analysis.

8.4.10 Survival and viability assessments

The number of shoots recovered and surviving in the recovery medium were assessed for each treatment for both frozen and unfrozen shoots after eight weeks of culture. Total recovery was defined as greening of shoot tips, leaf expansion and new shoot production.

The viability test using Triphenyl Tetrazolium Chloride (TTC) was also carried out for control (un-treated) and frozen shoot-tips (modified Steponkus and Lanphear, 1967). The shoot-tips were weighed to 100 ± 10mg (fresh weight), placed in test tubes (17 x 120 mm) and mixed with 3.0 ml of 0.6% (w/v) TTC in 0.05 M $Na_2HPO_4KH_2PO_4$ buffer (pH 7.4) + 0.05% (v/v) wetting agent (Tween 20). The samples, were then incubated at 28°C for 20 hours in the dark. After the incubation period, the TTC solution was drained and rinsed once with distilled water. The samples were extracted with 7 ml of 95% (v/v) ethanol for four minutes in the boiling water bath. The extracts were cooled, and made up to a 10 ml volume with 95% ethanol. The samples were mixed thoroughly and centrifuged at 500-1000g to remove cell debris. One ml of control sample, was added to a cuvette and absorbance measured using a spectrophotometer at wavelength 490 nm. This value was used as the control (initial) absorbance for subsequent samples. The above steps were repeated for frozen samples. The formazan produced in the tissue samples was expressed as a percentage of the amount produced (absorbance 490nm/g fresh weight) by unfrozen control samples.

8.5 Results

8.5.1 DSC

The DSC thermograms provided valuable information on critical cryopreservation parameters such as melt onset temperature, the enthalpy of melting and the amount of frozen (osmotically active) and unfrozen (osmotically inactive) water across the range of treatments investigated in this experiment. The average proportion of osmotically active water compared with the total water content was about 86% for control untreated shoot-tips. It declined to about 59% after 2.5% trehalose and 30 min PVS2 treatment. This average further declined to about 48% and 22% after 5 and 10% trehalose preculture respectively also with 30 min PVS2 treatment (Figure 8.3; Table 8.2). No osmotically active water content was detected for treatments with 60 and 90 min PVS2 treatment for all three trehalose concentrations. Likewise, the average proportion of osmotically inactive water compared with the total water content increased to 41, 52 and 78% after 2.5, 5 and 10% trehalose preculture respectively with 30 min PVS2 treatment. After 60 and 90 min PVS2 treatments the average reached a maximum of 100% (Figure 8.3; Table 8.2).

Representative cooling and warming thermograms are shown for control (no trehalose or PVS2 treatments) shoot-tip encapsulated in an alginate bead (Figure 8.3). Ice crystallization peaks (exothermic event with enthalpy variation 176.51 ± 8.78 $J.g^{-1}$; Table 8.3) during cooling and melting (endothermic event with enthalpy variation 150.73 ± 2.60 $J.g^{-1}$) during the warming cycle are shown in these thermograms respectively. The ice nucleation onset temperature, midpoint and endpoint data are summarized in Table 8.2.

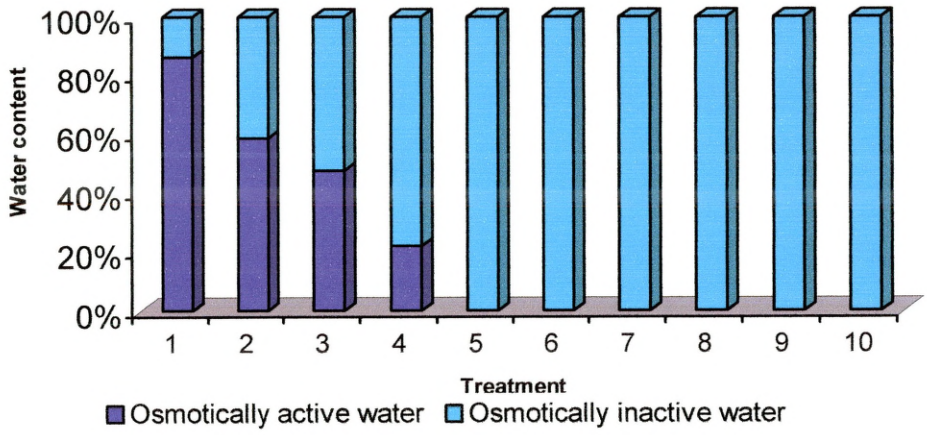


Figure 8.3. Percentage of osmotically active and inactive water contents as compared to total water contents

Note: Treatment 1= control, treatments 2-4 = 30 min PVS2, treatments 5-7 = 60 min PVS2, treatments 8-10 = 90 min PVS2. The treatment numbers are corresponds to those in Table 8.2.

Table 8.2. Water content of alginate-encapsulated shoot-tips of *P. speciosa*, followed by trehalose preculture and PVS2 treatment. Mean and standard errors (SE) of the means are displayed

Treatment	**Water Content (%FW)**	**Water Content (gg^{-1} DW) (Mean ± SE)**	**Osmotically Active Water Content[1] (gg^{-1} DW)**	**Osmotically Inactive Water Content[2] (gg^{-1} DW)**
1) Control	85.9 ± 0.3	6.068 ± 0.154	5.238 ± 0.105	0.830 ± 0.049
2) 2.5% Trehalose + 30 min PVS2	56.5 ± 1.7	2.305 ± 0.095	1.351 ± 0.083	0.954 ± 0.012
3) 5% Trehalose + 30 min PVS2	44.2 ± 4.5	1.371 ± 0.862	0.654 ± 0.158	0.717 ± 0.07
4) 10% Trehalose + 30 min PVS2	26.9 ± 1.9	0.368 ± 0.035	0.081 ± 0.003	0.287 ± 0.032
5) 2.5% Trehalose + 60 min PVS2	20.5 ± 1.9	0.258 ± 0.030	Not detected	0.258 ± 0.030
6) 5% Trehalose + 60 min PVS2	17.1 ± 1.3	0.207 ± 0.020	Not detected	0.207 ± 0.020
7) 10% Trehalose + 60 min PVS2	9.9 ± 0.7	0.110 ± 0.008	Not detected	0.110 ± 0.008
8) 2.5% Trehalose + 90 min PVS2	6.7 ± 1.5	0.172 ± 0.017	Not detected	0.172 ± 0.017
9) 5% Trehalose + 90 min PVS2	4.1 ± 0.3	0.094 ± 0.003	Not detected	0.094 ± 0.003
10) 10% Trehalose + 90 min PVS2	3.7 ± 1.4	0.130 ± 0.015	Not detected	0.130 ± 0.015

[1] Calculated by dividing the melt endotherm peak area (J) by 334.5 $J.g^{-1}$; 1g of water releases/absorbs 334.5 J of energy during the transition between liquid and ice phase (Block, 2003)

[2] calculated as the difference between total and osmotically active water contents

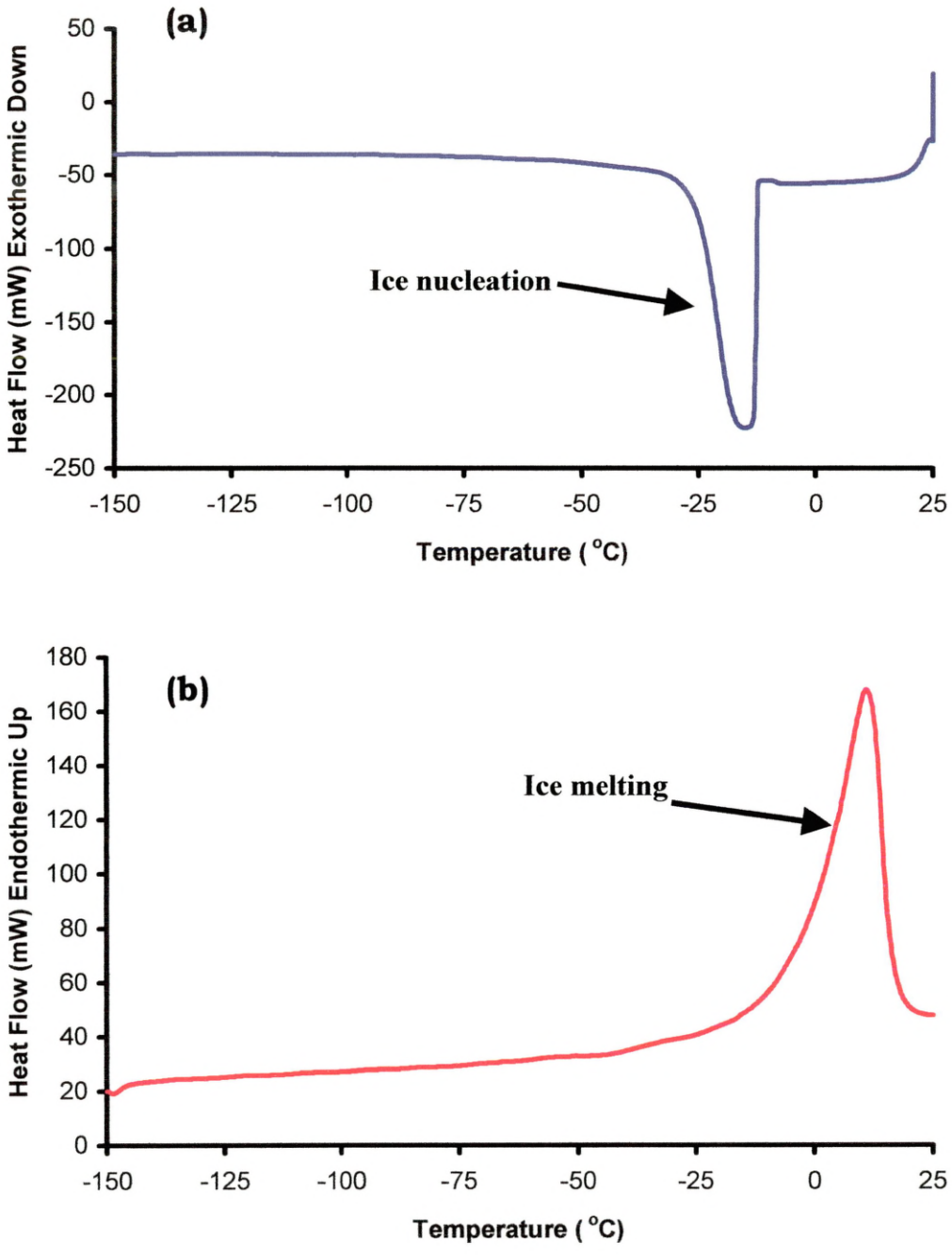


Figure 8.4. DSC cooling (a) and heating (b) thermograms for control (untreated) alginate-encapsulated *P. speciosa* shoot-tips. Samples were held at 25°C for 1 min and cooled to –150°C, held for 1 min and warmed to 25°C at a rate of 10°C per min.

The enthalpy of the melt endotherm varied in proportion to the water content (% FW) of the shoot-tips over concentrations of trehalose for 30 min PVS2 treatment (Table 8.4 and Figure 8.5b). The melt enthalpy for the control shoot-tip was 150.73 ± 2.60 $J.g^{-1}$. As the bead water content decreased with 2.5% trehalose the enthalpy was reduced to 61.38 ± 4.53 $J.g^{-1}$ and for 5% and 10% trehalose the enthalpy was further declined to 24.67 ± 1.04 and 12.29 ± 0.03 $J.g^{-1}$ respectively.

For shoot-tips treated with 2.5% trehalose and 30 min PVS2, no ice nucleation but a glass transition event (Tg with midpoint: -42.795 ± 2.49°C) was noted (Table 8.3 and Figure 8.5) during cooling indicating that a vitrified stage was achieved. However, ice melting was observed for this treatment during rewarming (enthalpy 61.38 ± 4.53 $J.g^{-1}$) indicating that the glass formed during cooling was not stable and it devitrified upon rewarming. Similar observations were noted for shoot tips treated with 5 and 10% trehalose with warming enthalpy 24.67 ± 1.04 and 12.29 ± 0.03 $J.g^{-1}$ respectively indicating glass (Tg) instability. More complex profiles were obtained with warming cycle. Depression of freezing point occur with 2.5% trehalose at –75°C and at –85°C with 5% trehalose (Figure 8.5b). There were also a number of minor thermal events which were in reverse to Tgs and were noted in almost all the treatment during cooling and warming cycles. The basis of these minor thermal events is not clear and it is possible that they could be attributed to glass relaxation events or localised thermal events with the cryoprotectants and plant matrix interaction which comprises a complex system. However, due to minor enthalpies and positions in the thermograms, they are not expected to have consequences to survival of the shoot-tips.

Increasing PVS2 treatment duration to 60 min, resulted in a more thermodynamically stable profile with no thermal event during cooling and a glass transition during subsequent rewarming with Tg midpoint: -33.24 ± 0.23, -31.89 and -34.78°C for 2.5, 5 and 10% trehalose respectively (Figure 8.6). Further increasing PVS2 treatment to 90 min completely eliminated the active water content (Table 8.2). This is evidenced by stable thermal profile during cooling and subsequent rewarming for all three trehalose concentrations with 90 min PVS2 treatment (Figure 8.7).

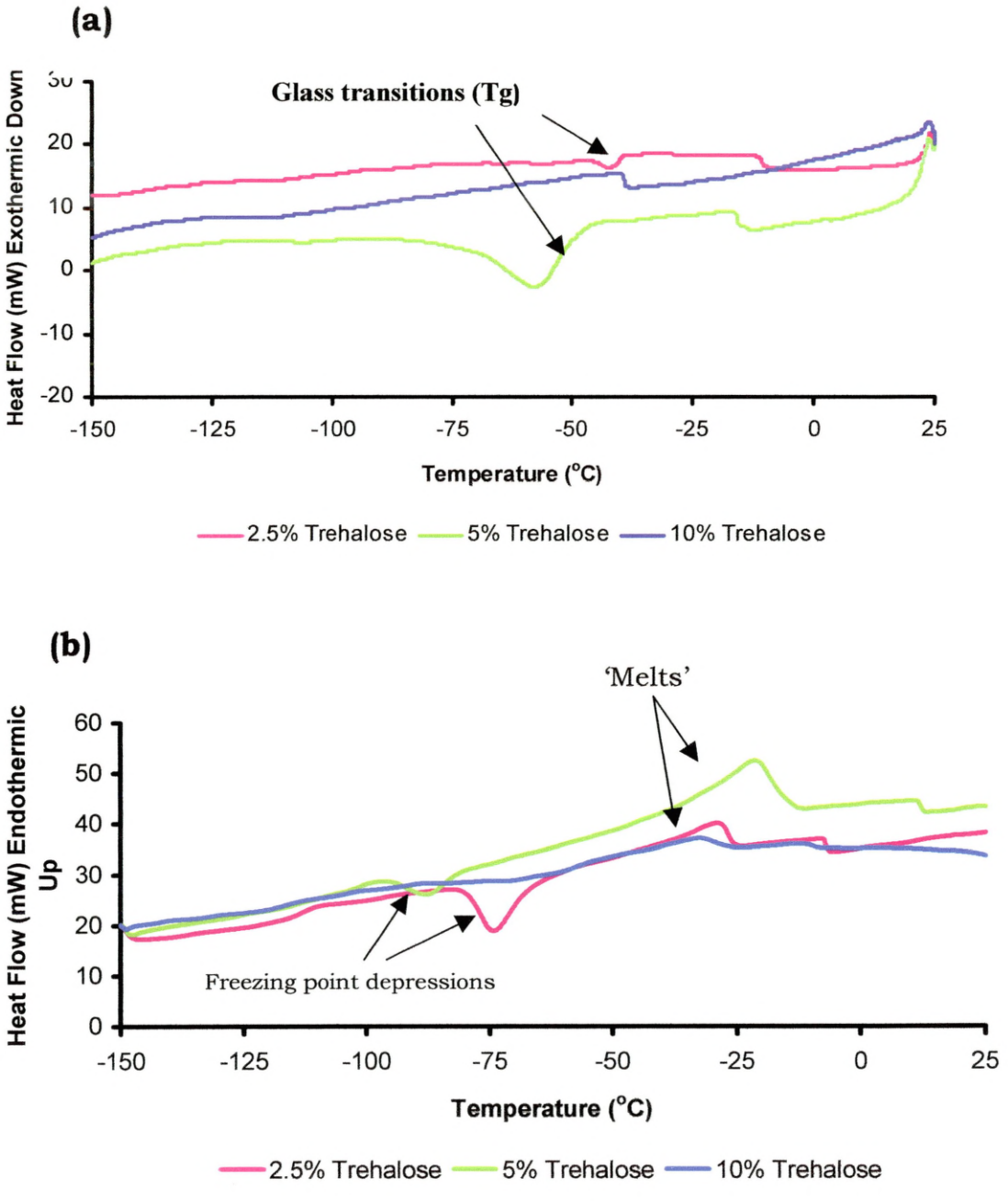


Figure 8.5. DSC cooling (a) and heating (b) thermograms for alginate-encapsulated *P. speciosa* shoot-tips treated with 2.5%, 5% and 10% Trehalose in pre-growth medium and treated with **PVS2 for 30 min.** Samples were held at 25°C for 1 min and cooled to –150°C, held for 1 min and warmed to 25°C at a rate of 10°C per min.

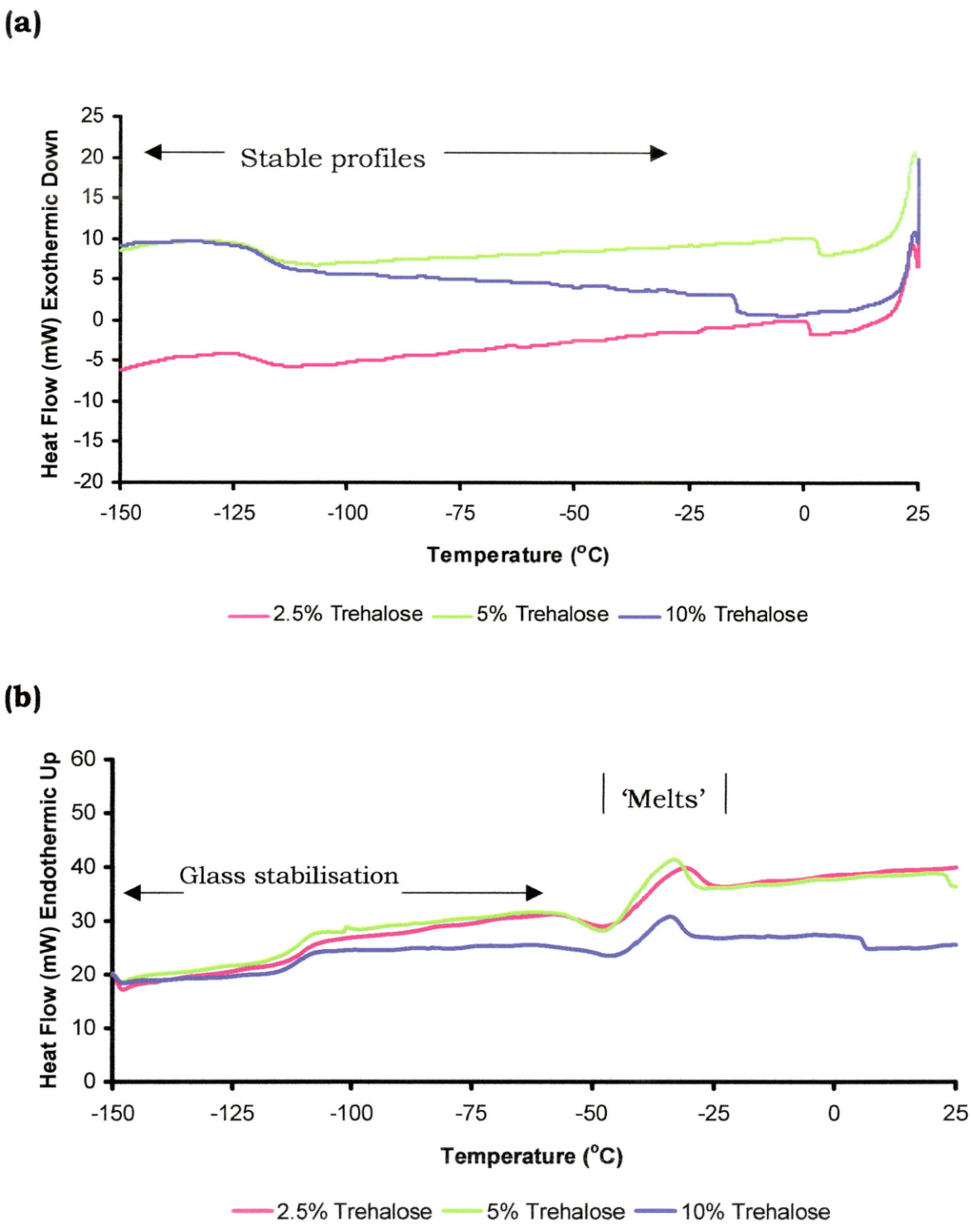


Figure 8.6 DSC cooling (a) and heating (b) thermograms for alginate-encapsulated *P. speciosa* shoot-tips treated with 2.5, 5 and 10% trehalose in pre-growth medium and followed by **60 min PVS2** treatment. Samples were held at 25°C for 1 min and cooled to –150°C, held for 1 min and warmed to 25°C at a rate of 10°C per min.

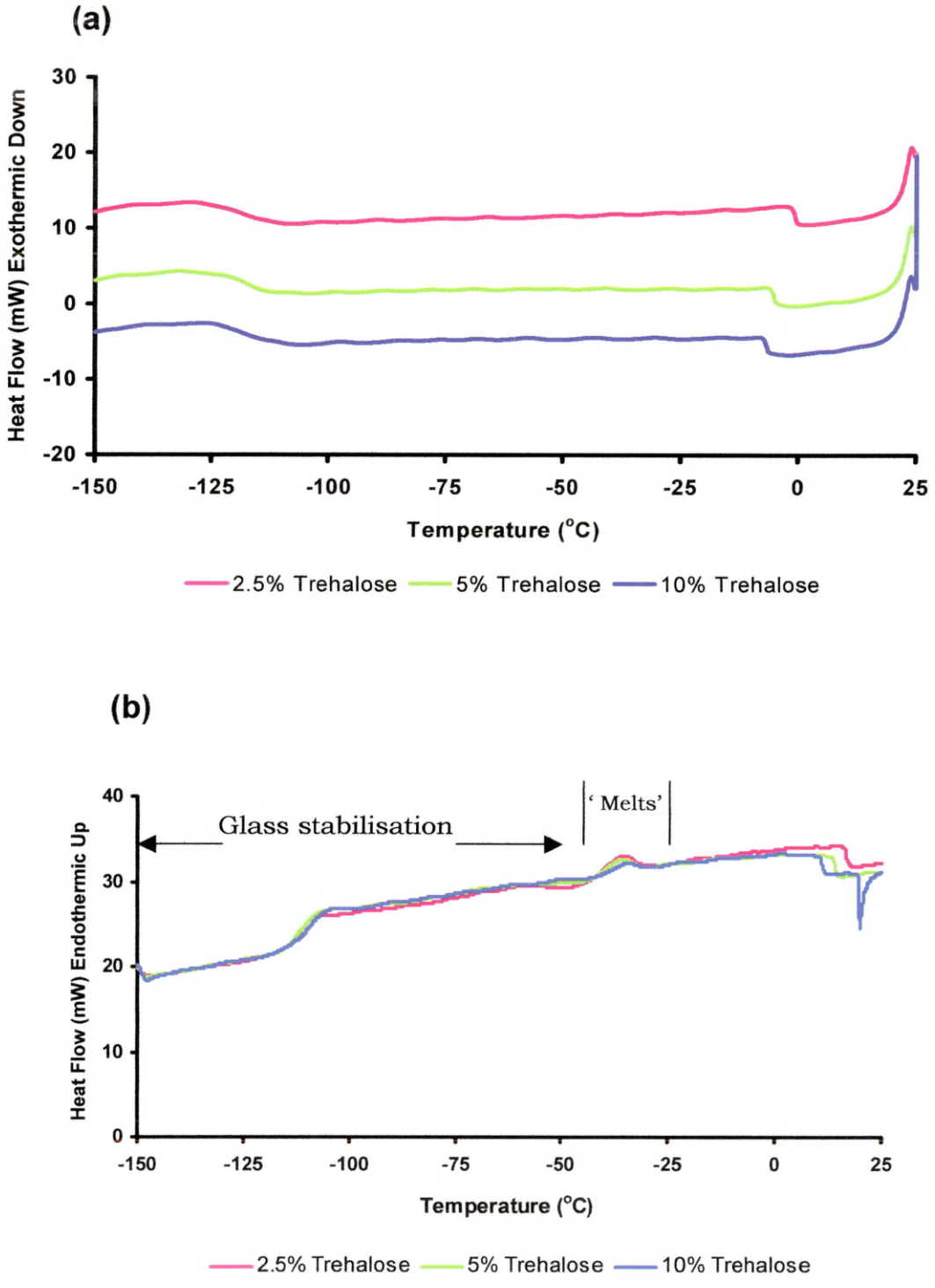


Figure 8.7 DSC cooling (a) and heating (b) thermograms for alginate-encapsulated *P. speciosa* shoot-tips treated with 2.5, 5 and 10% trehalose in pre-growth medium and followed by **90 min PVS2** treatment. Samples were held at 25°C for 1 min and cooled to –150°C, held for 1 min and warmed to 25°C at a rate of 10°C per min.

Table 8.3. Thermodynamic cooling properties of alginate-encapsulated shoot-tips of *P. speciosa*, following trehalose preculture and PVS2 treatment. Means ± standard errors of the mean are displayed

Trehalose (%) in pre-growth medium	PVS2 (min)	Thermal Event[1]	Onset (°C)	Mid-point (°C)	Endpoint (°C)	Enthalpy ($J.g^{-1}$)	Heat Capacity ($J.g^{*o}C^{-1}$)
Control	0	Ice nucleation*	-13.80 ± 1.48	-16.66 ± 1.49	26.16 ± 1.14	176.51 ± 8.78	NA
2.5	30	Tg*	-40.22 ± 0.95	-42.79 ± 2.49	-44.75 ± 3.72	NA	0.17 ± 0.05
5.0	30	Tg*	-50.21 ± 1.07	-54.25 ± 0.93	-57.32 ±0.91	NA	0.23 ± 0.01
10.0	30	θ	NA	NA	NA	NA	NA
2.5	60	θ	NA	NA	NA	NA	NA
5.0	60	θ	NA	NA	NA	NA	NA
10.0	60	θ	NA	NA	NA	NA	NA
2.5	90	θ	NA	NA	NA	NA	NA
5.0	90	θ	NA	NA	NA	NA	NA
10.0	90	θ	NA	NA	NA	NA	NA

* = event occur in both replicates; θ = stable profile, no event detected in either replicates

Table 8.4. Thermodynamic warming properties of alginate-encapsulated shoot-tips of *P. speciosa*, following trehalose preculture and PVS2 treatment. Means and standard errors of the mean are displayed

Trehalose (%) in pre-growth medium	PVS2 (min)	Thermal Event[1]	Onset (°C)	Midpoint (°C)	Endpoint (°C)	Enthalpy ($J.g^{-1}$)	Heat Capacity ($J.g^{*o}C^{-1}$)
Control	0	Ice melt*	-1.66 ± 0.13	10.57 ± 0.46	16.30 ±0.18	150.73 ± 2.60	NA
2.5	30	Ice melt*	-39.56 ± 0.06	-32.54 ± 0.04	-27.31 ± 0.21	61.38 ± 4.53	NA
5.0	30	Ice melt*	-35.56 ± 0.26	-21.87 ± 0.24	-15.03 ± 0.94	24.67 ±1.04	NA
10.0	30	Ice melt*	-38.73 ± 0.45	-29.01 ± 0.18	-26.00 ± 0.11	12.29 ± 0.03	NA
2.5	60	Tg*	-39.57 ± 0.43	-33.24 ± 0.23	-29.70 ± 0.31	NA	1.88 ± 0.21
5.0	60	Tg#	-40.14	-31.89	-27.72	NA	1.57
10.0	60	Tg#	-45.04	-34.78	-30.67	NA	1.06
2.5	90	θ	NA	NA	NA	NA	NA
5.0	90	θ	NA	NA	NA	NA	NA
10.0	90	θ	NA	NA	NA	NA	NA

* = event occurs in both replicates; # = event occurs in one replicate out of two; θ = stable profile, no event detected in both replicates.

8.5.2 Survival

Survival was assessed, as the number of shoots that remained green, producing new leaves and shoots (Figure 8.8). Binary logistic regression analysis showed that fitting trehalose and its quadratic was not significant ($G^* = 1.297$, df = 2, Model 2, Table 8.5). This suggests, that all the trehalose levels produces the same rate of survival. Above 70% survival was achieved after trehalose pre-treatment regardless of preculture duration (1, 2 and 3

days) before cryopreservation. This showed that trehalose at 2.5, 5 and 10% does not have detrimental effect on the shoot-tips of *Parkia speciosa.*

Fitting the full quadratic model involving trehalose and preculture time also does not have a significant effect on the shoot-tips survival before cryopreservation ($G^* = 4.612$, df = 1; $p>0.05$, Model 3; Table 8.5) compared to model 2. This indicates that all the three preculture times (1-3 days) and trehalose levels have a similar effect on the survival of the shoot-tips. However, adding PVS2 treatment and its quadratic to the model (Model 4, Table 8.5), had a highly significant effect on the model predicting the survival ($G^* = 96.795$, df = 2, $p<0.001$) compared to model 3, suggesting that there is a significant difference in shoot-tips survival before and after PVS2 treatment and all the three levels of PVS2 treatment have different effect on model predicting survival. Fitting the full model involving trehalose, preculture duration and PVS2 treatment and their interactions does not have a significant effect on the shoot-tips survival. Survival before cryopreservation was around 90% for 30 min PVS2 treatment and decreased to about 70% with 60 min PVS2, and then decreased drastically to an average of (0-10%) with 90 min PVS2 treatment. This result shows that increasing PVS2 treatment to 90 min is detrimental to the shoot-tips.

Adding LN to the above model (Model 5, Table 8.5), significantly improves the model predicting survival ($G^* = 102.169$, df = 1, $p<0.001$) compared to model 4, showing that there is a significant difference on the survival of the shoot-tips before and after cryopreservation. The clear difference in survival before and after cryopreservation was noted for shoot-tips treated with 30 min PVS2 where the survival before LN storage was above 70% but declined to about 20% after cryopreservation. Fitting the full quadratic model involving trehalose, preculture duration, PVS2 treatment together with LN and their interactions did not have significant effect on the model predicting survival of the shoot-tips.

Table 8.5. Analysis of deviance for trehalose pre-treatment, PVS2 and liquid nitrogen storage for *Parkia speciosa* shoot-tips survival

Model	**Deviance explained, G**	**DF**	**Deviance difference, G***	**DF difference**	**P value**
1. Null	0	0			
2. $T + T^2$	1.297	2	1.297	2	>0.05
3. $T + T^2 + D + D^2 + T*D$	5.909	5	4.612	3	>0.05
4. $T + T^2 + D + D^2 + T*D + PVS2 + PVS2^2$	102.704	7	96.795	2	**<0.001**
5. $T + T^2 + D + D^2 + T*D + PVS2 + PVS2^2 + LN$	204.873	8	102.169	1	**<0.001**

T=Trehalose; T^2 = quadratic function of T; D=preculture duration, D^2 = quadratic function of D; $PVS2^2$= quadratic function of PVS2; LN=liquid nitrogen.

8.5.3 Viability assay

Viability was shown in the amount of formazan (per gram dry weight) produced by the cryopreserved shoot-tips (after trehalose and PVS2 treatments) following TTC solution incubation and was expressed as a percentage of the total amount of TTC reduction by the unfrozen control shoot-tips. The percentage of formazan produced was about 94% for non-cryopreserved control shoot-tips (Table 8.6). All shoot-tips treated for 60 min PVS2 had produced formazan above 55% regardless for percentage trehalose and time of preculture. Shoot-tips precultured with 5% trehalose for 2 days followed by 60 min PVS2 treatment produced the highest amount of formazan (89.55 ± 7.48%), which showed the closest value to the control shoot-tips. The percentage formazan produced was low for 30 min PVS2 treatment followed by 90 min PVS2 treatment (Table 8.6). Table 8.6 summarises the results for survival before and after cryopreservation and the TTC assay after cryopreservation for all the treatments investigated. The TTC staining intensity varied between cell types. Meristematic cells were more intensely stained compared to the distal cells (Figure 8.9).

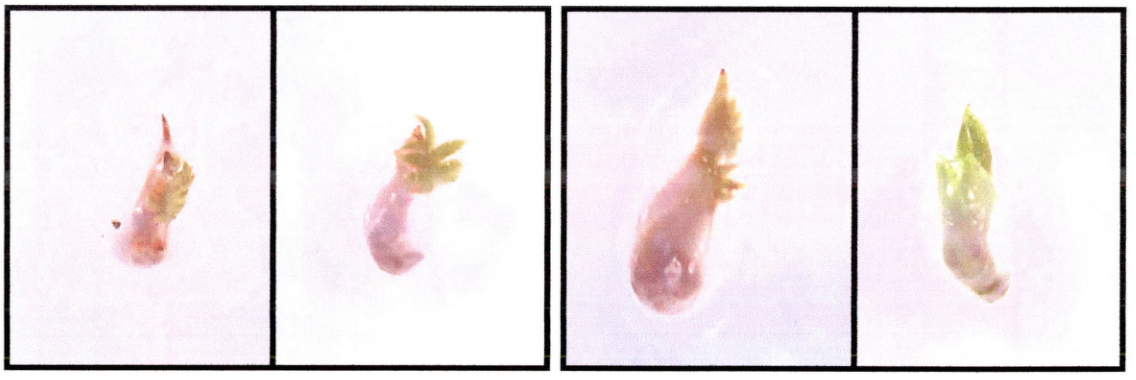


Figure 8.8. Cryo-storage survival of *P. speciosa* shoot-tips after eight weeks of culture in recovery medium

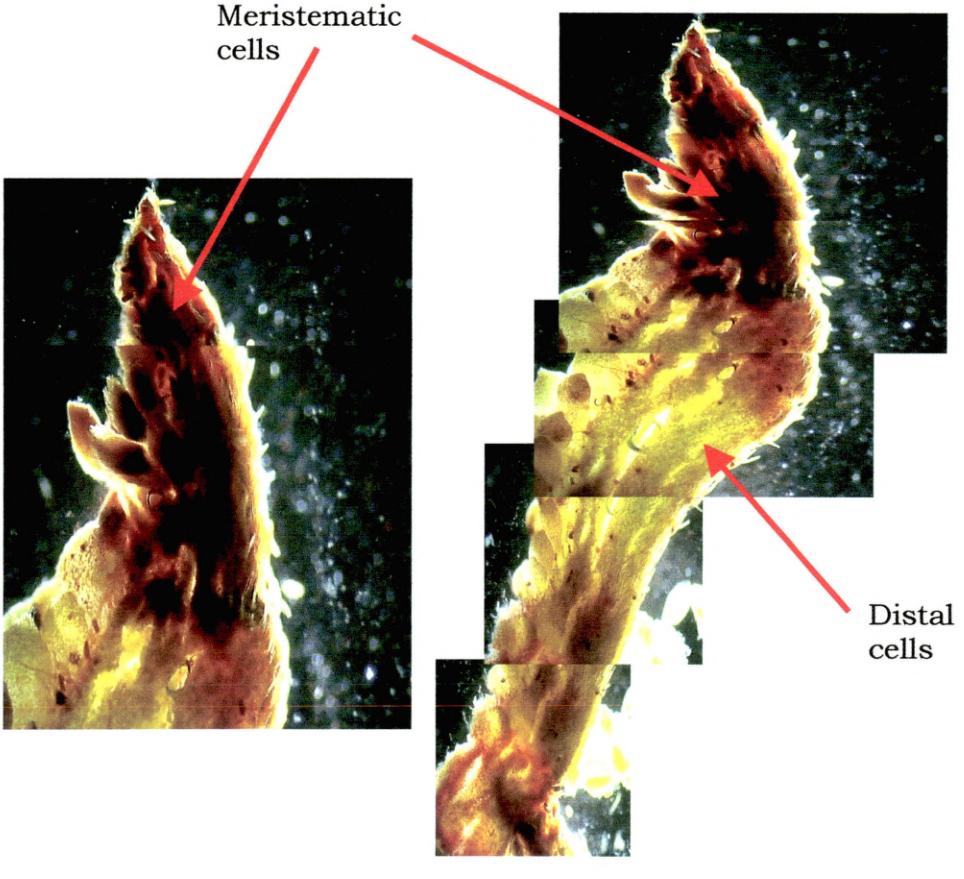


Figure 8.9. Cryopreserved *Parkia speciosa* shoot-tips after Triphenyl Tetrazolium acetate (TTC) viability staining assay

Table 8.6 Survival before and after cryopreservation and viability after cryopreservation (± standard error) of *P. speciosa* shoot tips after various trehalose and PVS2 treatments

	Treatment			Survival (%)		Viability
Treat-ment Number	**Trehalose (%) in pre-growth medium**	**Pre-growth duration (day)**	**PVS2 (min)**	**-LN**	**LN**	*** Formazan produced (%)**
1	control	0	0	96.7 ± 5.8	0 ± 0	94.4 ± 2.8
2	2.5	1	30	86.7 ± 5.8	30.7 ±10.0	52.8 ± 6.9
3	2.5	1	60	66.7 ± 5.8	56.7 ± 5.8	72.8 ± 5.0
4	2.5	1	90	0.0 ± 0.00	0.0 ± 0.00	19.0 ± 8.4
5	2.5	2	30	66.7 ± 5.8	6.7 ± 5.8	48.8 ± 4.9
6	2.5	2	60	66.7 ± 5.8	60.0 ± 10.0	64.4 ± 8.8
7	2.5	2	90	6.7 ± 5.8	0.0 ± 0.0	10.4 ± 0.9
8	2.5	3	30	70.0 ± 10.0	13.3 ± 5.8	46.5 ± 9.2
9	2.5	3	60	76.7 ± 15.3	53.3 ± 15.3	64.9 ± 12.2
10	2.5	3	90	6.7 ± 5.8	0.0 ± 0.0	23.2 ± 8.6
11	5	1	30	60.0 ± 10.0	26.7 ± 5.8	47.4 ± 11.2
12	5	1	60	90.0 ± 10.0	70.0 ± 10.0	87.0 ± 8.2
13	5	1	90	0.0 ± 0.0	0.0 ± 0.0	14.9 ± 2.4
14	5	2	30	80.0 ± 10.0	20.0 ± 10.0	40.3 ± 4.5
15	5	2	60	80.0 ± 10.0	66.7 ± 5.8	89.6 ± 7.5
16	5	2	90	10.0 ± 10.0	3.4 ± 5.8	16.7 ± 7.1
17	5	3	30	70.0 ± 0.0	26.7 ± 5.8	55.1 ± 9.4
18	5	3	60	80.0 ± 10.0	80.0 ± 10.0	84.2 ± 13.0
19	5	3	90	10.0 ± 10.0	6.7 ± 5.8	24.7 ± 10.2
20	10	1	30	76.7 ± 5.8	23.4 ± 5.8	20.3 ± 12.0
21	10	1	60	76.7 ± 11.6	60.0 ± 10.0	56.6 ± 6.0
22	10	1	90	0.0 ± 0.0	0.0 ± 0.0	15.9 ± 9.0
23	10	2	30	70.0 ± 10.0	20.0 ± 10.0	30.1 ± 5.3
24	10	2	60	70.0 ± 0.0	63.4 ± 5.8	87.9 ± 10.1
25	10	2	90	6.7 ± 5.8	0.0 ± 0.0	16.9 ± 6.3
26	10	3	30	70.0 ± 10.0	23.4 ± 5.8	31.7 ± 6.6
27	10	3	60	60.0 ± 10.0	63.4 ± 5.8	55.6 ± 10.3
28	10	3	90	0.0 ± 0.0	0.0 ± 0.0	12.3 ± 4.1

*Percentage formazan produced per dry weight of cryopreserved shoot tips as compared to the non-cryopreserved control shoot tips

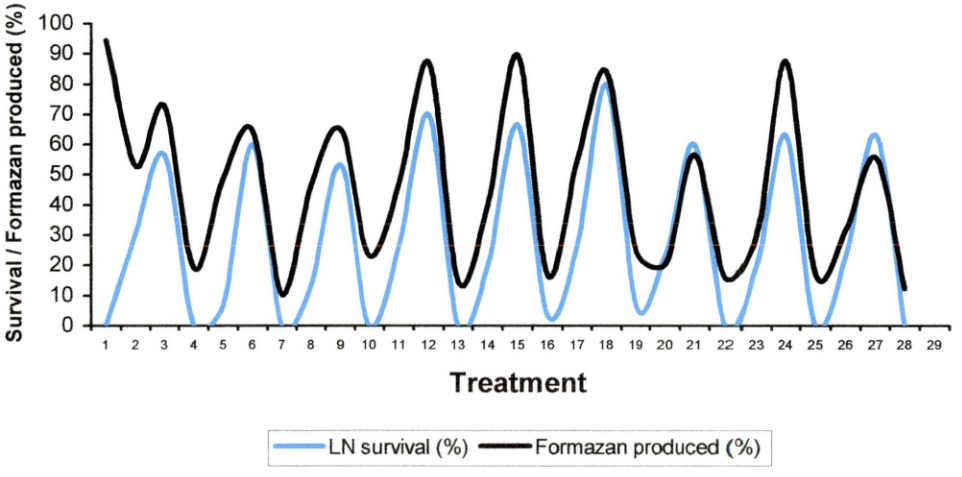


Figure 8.10. Percentage survival and formazan production cryopreserved shoot-tips after various cryoprotectant treatments

(Note: The treatment numbers are the numbers assigned to each different combination of cryoprotective treatment as used in Table 8.6)

8.6 Discussion

Thermal analysis using DSC was carried out during cooling and warming cycles of alginate encapsulated shoot tips after various pre-treatments as summarised in Table 8.1. Clear evidence of ice nucleation during cooling and ice melting during warming were noted for control shoot tips (Figure 8.4a and 8.4b). These thermograms indicate clear evidence of the presence of excess water which formed ice crystal during freezing and warming which was detrimental to the shoot tips evidenced by zero post-cryopreservation survival (Table 8.6).

8.6.1 PVS2 treatment for 30 min

In this study, vitrification evidenced by a glass transition was achieved after 30 min PVS2 treatment regardless for trehalose concentration in the preculture media. However, more complex profiles were obtained with the warming cycle, where an endothermic event was detected suggesting glass destabilization for this treatment (Figure 8.5b). This event is known as devitrification which describes that during cooling the sample attains the glassy state but it invariably is meta-stable. It may contain ice nuclei, the growth of which is arrested along with all other molecular motions in the sample. However, upon rewarming crystallization can be detected by an exothermic event often below 0°C in a thermogram, reflecting the growth of ice by devitrification (transition of glassy to crystalline state) and recrystallization (growth of existing ice crystals) (Taylor *et al.*, 2004). This reflects the high probability that the vitreous system containing ice nuclei and if warming is not sufficiently rapid, further nucleation and crystallization will occur resulting in lethal ice-damage. The phenomenon of crystallization on warming a glassy sample to temperatures in the vicinity of Tg is often referred to as devitrification of a doubly unstable glass since it is unstable with respect to both the liquid and solid states (Mazur, 2004). Dumet *et al.*, (2000b) reported a glass destabilization event upon rewarming of 4 hr desiccated alginate encapsulated *Ribes ciliatum* meristems. However, they noted no glass transition in the alginate bead or meristem when these were cooled or rewarmed separately. They postulated that it could be the consequence of the differences between the thermal properties of the alginate bead and the meristem, which might have promoted the ice

nucleation. It was also suggested that a differential moisture gradient might exist between the tissue and the bead, which could promote glass destablization events on rewarming.

8.6.2 PVS2 treatments for 60 and 90 min

In this study, a vitrified state was achieved as evidenced by the Tg event after 60 min PVS2 treatment upon cooling and subsequent rewarming regardless of the trehalose concentration in the preculture media (Figure 8.6). This shows that a longer PVS2 treatment could eliminate ice crystallization during cooling and devitrification during subsequent rewarming. Avoidance of ice by vitrification was achieved by cooling highly concentrated solutions that becomes sufficiently viscous at low temperatures to suppress crystallization. PVS2 treatment for 90 min completely removed all active water (Table 8.2) based on the detection limits of this system. This was also shown in the DSC thermograms where a stable profile with no thermal events recorded for this treatment upon cooling and following rewarming procedure (Figure 8.7). Some 'melting' events were noted for 60 min PVS2 treatment during the warming cycle as shown in the thermogram (Figure 8.7b). These could be a devitrification process, however, the scale is too small and when related to survival data, these events do not have effect on the survival since high survival rate was noted for 60 min PVS2 treatment. Some small scale 'melting' events were still noted for 90 min PVS2 treatment, they were much suppressed compared to 60 min PVS2.

This study showed how DSC can assist in developing cryopreservation protocols for recalcitrant seeds, where it very accurately measures the nature and intensity of thermal events during a freeze-thaw cycles. An increase in survival is systematically associated with a decrease in the intensity of ice crystallization/ ice melting events and optimal survival is usually reached when only vitrification events are recorded (Hor *et al.*, 2005; Dussert *et al.*, 2001; Block, 2003; Dumet *et al.*, 2000b; Kim *et al.*, 2005). Thermal analysis provides a fundamental approach to improve the current understanding of recalcitrant species. Benson *et al.* (2004) applied DSC thermal analysis to optimise critical points of the cryoprotective strategies and thermal behaviours of encapsulated/dehydrated somatic embryos from

the tropical, medicinal tree, neem (*Azadirachta indica* A. Juss). DSC analysis was performed after encapsulation, 24 h dehydration in 0.3, 0.5, 0.7 M sucrose, and following airflow desiccation for 1, 2, 3 and 4 h. As the cryoprotective treatment was progressed through subsequent evaporative desiccation steps, exotherm size diminished to a point at a critical time (3 h) at which nucleation was inhibited and a vitrified state formed. Similarly, the magnitude of the endotherm melt decreased with increasing desiccation time. This exemplifies how DSC may be applied as an investigative tool to help formulate new cryoprotection strategies for limited-access, rare and at risk tropical germplasm that has never been cryo-conserved before.

8.6.3 Trehalose preculture treatment

P. speciosa shoot-tips were treated with 2.5, 5 and 10% trehalose in the preculture medium for one to three days prior to cryopreservation.

Some exceptional properties of trehalose have been compared with other sugars as cryo-protectant by many researchers. It was reported that the glass transition temperature (Tg) for trehalose is much higher than that for sucrose, fructose and glucose (Green and Angell, 1989; Wang and Haymet, 1998). Glass transition temperature is an important parameter reflecting the stability of a material (Yoshii *et al.*, 2000). In this study, for 30 min PVS2 treatments, the Tg was lower (-50.21 ± 1.07°C) for 2.5% trehalose and it increased to -40.22 ± 0.95°C as the trehalose concentration was increased to 5% (Table 8.3, Figure 8.5a). A glass transition is also associated with the mobility of molecules within samples (Yoshii *et al.*, 2000). In this study, the heat capacity of the glass transition for shoot-tips treated with 2.5% and 5% trehalose for 30 min PVS2 (Figure 8.5a; Table 8.3) increased slightly from 0.17 ± 0.05 to 0.23 ± 0.01 $J.g^{*\circ}C^{-1}$ respectively. The glass transition peak shown by the 5% trehalose pre-treatment might indicates that the water molecules in this sample treated with higher concentration (5%) of trehalose might have less mobility compared to those treated with 2.5% trehalose. This maybe due to the strong interaction or binding between trehalose and water molecules. Bordat *et al.*, (2004), showed that trehalose has superior effects in de-structuring the matrix network of water and in slowing down its matrix dynamics. These two properties could play a key role in the understanding of the molecular mechanisms of bioprotection of

trehalose. Sakurai *et al.*, (1997) reported that in trehalose, all of the hydroxyl groups can act as both a proton donor and an acceptor in forming hydrogen bonds with water, accounting for the superior hydration ability and biological function of this sugar.

This study showed, that the enthalpy of the melt-endotherm varied in proportion to the concentrations of trehalose for 30 min PVS2 treatment (Table 8.4 and Figure 8.5b). The melt enthalpy for the control shoot-tips was above 150 $J.g^{-1}$. and it decreased to around 60 $J.g^{-1}$ with 2.5% trehalose and with 5% and 10% trehalose the enthalpy further declined to about 24 and 12 $J.g^{-1}$ respectively. Freezing points were depressed to –75°C and –85°C with 2.5% and 5% trehalose respectively (Figure 8.5b). This result showed that trehalose treatment does have a considerable effect on the thermal events of the shoot-tips. Wang and Haymet (1998) suggested that trehalose in aqueous solution showed less freezing of water molecules compared with sucrose reflected by the fact that the trehalose solution shows the lowest heat of freezing and heat of melting. On the other hand, both the high melting point and a higher glass transition temperature and its effective in cryopreservation suggests that it is the unique structural features of trehalose that are responsible for all the above differences (De Carlo *et al.*, 1999). This might further explain the post-cryopreservation survival obtained for *P. speciosa* shoot-tips in this study. It may be worthwhile to explore the application of trehalose in tropical recalcitrant germplasm cryopreservation in the future.

8.6.4 Post-cryopreservation survival

DSC thermal profiles provide critically important information about the cryoprotective treatments required to obviate lethal ice formation and stabilize glasses. In this study, the DSC thermograms confirmed a less stable glass transition at 30 min PVS2 treatment, followed by a stable glass transition at 60 min PVS2 and a stable profile with no thermal events at 90 min PVS2 treatments. PVS2 treatment for 90 min completely removed all the osmotically active water (Table 8.2). However, when this is related to post-cryopreservation survival and viability data, very low (20%) survival was achieved after 30 min PVS2 treatment, 60% survival after 60 min PVS2

treatment and no survival was recorded after 90 min PVS2 treatment regardless for trehalose pre-treatment. This explains that 30 min PVS2 treatment was not sufficient to eliminate most of the active water and glass destabilization on rewarming, which might have caused additional damage to shoot tips and consequently reduced survival after cryopreservation. PVS2 treatment for 90 min though completely removed all osmotically active water, no survival was recorded. This is probably due to high phyto-toxicity of the PVS2 solution or the shoot-tips are extremely sensitive to removal of osmotically active water as reported by (Vertucci *et al.*, 1991; Benson *et al.* 1996b; Dereuddre *et al.*, 1991; Dumet *et al.*, 2000b; Hor *et al.*, 2005). Benson *et al.*, (2004), recommended that for these systems, the possibility that limited intracellular ice nucleation may be tolerated is contentious as tissues may potentially survive limited intra-cellular ice formation if the ice crystals formed are too small to cause significant damage.

The formation of a stable glassy state during cooling and warming achieved with the 60 min PVS2 treatment is a prerequisite for post-cryopreservation survival of *P. speciosa* shoot-tips.

8.6.5 Post-cryopreservation viability

TTC reduction, as utilized in the present study, is the most convenient and quickest method for multiple cell viability assessments (Sadia *et al.*, 2003). In this study, however, TTC assessment results alone did not gave an adequate interpretation of viability since some absorbance values were recorded by dead shoot-tips which were treated with 90 min PVS2. Therefore, besides the TTC assessment, survival of shoot-tips was also assessed after rewarming as recommended by Ishikawa *et al.*, (1995). The survival data were consistent with the data obtained from TTC reduction assessments. It was also noted, that the TTC staining intensity varied between cell types in the shoot tips. The meristematic cells which are actively dividing showed instance staining compared to the distal cells (Figure 8.8).

8.7 Conclusions

- Trehalose preculture treatment has a considerable effect on the thermal events in cryopreserved *P. speciosa* shoot-tips. However, the logistic regression analysis showed that only PVS2 treatment had a significant effect on the survival of the shoots with highest post-cryopreservation survival recorded for 60 min PVS2 treatment regardless of trehalose concentration and preculture time tested in this experiment.

- PVS2 treatment for 60 min was optimal with stable glass formation during cooling and warming as evidenced by the DSC thermal analysis. A 30 min PVS2 treatment was not sufficient to produce a stable glass and zero post-cryopreservation survival after 90 min PVS2 treatment could be too toxic or the shoot tips were extremely sensitive removal of all active water.

8.8 Recommendations For Future Recalcitrant Seed Cryopreservation

As mentioned earlier, this chapter took a different perspective compared to previous chapters owing to the recalcitrance of the species studied. The integrated approaches which include:

1. Increase the availability of germplasm by using microprogapation to avoid seed limitation problem,
2. Applying thermal analysis using DSC to evaluate critical steps in the cryopreservation protocol and
3. Applying of novel cryoprotectant, trehalose in the preculture medium

These approaches are expected to provide the seed technologist working with the cryopreservation of recalcitrant germplasm a fundamental knowledge of this system. Having acquired the above knowledge, the future experiments on this species could progress to include the application of statistical tools, i.e. efficient experimental designs using factional factorial or Taguchi methods. These experimental designs require pre-knowledge of the basic storage behaviour of germplasm and their application is therefore cautioned in the absence of available of a species. In particular, full

experiments were not able to be carried out due to limitation of germplasm supply. A suggested future experiment for *P. speciosa* species could be using micropropagated shoot-tips with further investigation on trehalose around 5% (e.g. 4.5%, 5% and 6%) and PVS2 treatment around 60 minutes (e.g. for 50, 60 and 70 minutes) since this levels seems to provide higher post cryo-survivals.

Having completed all the experiments on orthodox, intermediate and recalcitrant seeds so far, the next chapter will discuss previous chapters in a final and general appraisal of the applications of novel statistical and investigated cryopreservation approaches for problematic tropical germplasm conservation.

CHAPTER 9

GENERAL DISCUSSIONS AND CONCLUSIONS

9.1 Cryopreservation Protocol Development

This study has explored the novel application of statistically designed experiments for the cryopreservation protocol development for problematic tropical forest tree germplasm. Incorporating a progressive study from orthodox, intermediate to recalcitrant seeds. New cryopreservation protocols have been developed for the orthodox and intermediate groups and the application of novel experimental designs has facilitated this end.

The recalcitrant seed problem has been addressed somewhat differently using *in vitro* micropropagation techniques and the application of cryophysical studies using DSC to help elucidate critical points of cryoprotective additive treatments. For the first time, trehalose has been explored as a possible cryoprotectant for tropical recalcitrant tree germplasm.

9.2 Experimental Designs and Data Analysis

Owing to the problems associated with obtaining sufficient seed material, the rapid ageing of tropical forest seeds and many other non-cryobiological conservation problems, cryopreservation protocol development for tropical forest tree species needs an integrated approach. It is therefore critical to develop an efficient experimental design, which allows the rapid screening of possibly many cryogenic factors without the need to sacrifice large numbers of seeds in the process of protocol development. A well-designed experiment prior to seed collection is essential taking into consideration the limitation of germplasm availability and the importance of conducting the experiments rapidly. By designing efficient experiments that take into account these considerations for cryopreservation protocol development, it will be possible to implement improved cryoprotection strategies. This is extremely important in the application of cryo-conservation for tropical species, which produce recalcitrant seeds in limited quantities and at restricted time intervals and have proved difficult to conserve.

In this thesis, for all the studies, full factorial experiments were conducted and suitable subsets were selected to simulate fractional replicates or orthogonal arrays. This allows the comparison of analyses and conclusions from the experiments as a full, fractional factorial and also as Taguchi designs. For example, in the first experiment with orthodox seed (Section 6.3), the full factorial experiment (32 treatment combinations) was conducted and suitable parts were selected to simulate fractional factorial experiment of 16 and 8 treatment combinations (half and quarter experiments) respectively and also as Taguchi L_{32}, L_{16} and L_8 designs.

9.2.1 Comparison of full and fractional factorial experiments at two-levels

The simplest design of a factorial experiment is two-level design, where each input variable is varied at high and low levels and the output observed for resultant changes (Box *et al.*, 1978). In this thesis, factorial experiments were used to investigate main effects and two-way interactions. The advantages of full factorial design include orthogonality, the ability to exclude aliasing and the evaluation of all main effects and interactions (Box *et al.*, 1978). In this thesis, all the full factorial experiments used were orthogonal and therefore, the interpretations of their analyses were not difficult. In the full factorial experiment none of the effects are aliased together therefore no information on the effects is lost using this design. All three-way or higher order interactions were excluded from the analyses according to the hierarchical order principle (Wu and Hamada, 2000). The disadvantages of full factorial experiments include time, cost and resource commitment. One main disadvantage of factorial experiment is the number of the treatment combinations which rises rapidly with the number of factors (Staines *et al.*, 1999). In this thesis, in Section 6.3, the full factorial experiment with five factors at two levels had 32 treatment combinations and since forty replicates (one seed in a single replicate) were used in each treatment combination, the whole experiment used 1280 seeds. This is not economical especially when there is an urge to conduct the cryopreservation experiments for tropical germplasm rapidly.

This is the first time, fractional factorial replication has been investigated for tropical tree species cryopreservation experiments. This study showed that it is not always necessary to run the full number of two-level combinations since the fractionally replicated experiments provided similar results to the full experiments. One advantage of fractional experiments is that they can be used to predict unobserved values without actually running the experiment. This reduces the number of observations and thus the experimental time compared to the original full experiment which is extremely useful for cryopreservation protocol development experiments. By making use of fractional designs, the two-level approach can be extended to many factors. Therefore, these designs are ideal for screening many factors to identify the (hopefully) fewer factors that significantly affect the responses. Fractional factorial designs retain orthogonality while requiring fewer runs. However, doing fewer runs means acquiring less information. To ensure that subsequent run capture the most important information, the insignificant higher order or all interactions are usually ignored (Wu and Hamada, 2000).

The disadvantage of fractional factorial experiment is the construction of the design itself. As described in Section A1.5, there are two important considerations for the construction of fractional factorial experiments; aliasing and resolution. The subsets of the full factorial experiment need to be selected carefully so that the important effects are not aliased. In this study, in the first experiment, a half fractional replicate of full factorial experiment was designed using aliasing structure I = ABCDE (resolution V). Therefore, no main effects and two-way interactions were aliased together. However, the quarter replicate was designed using aliasing structure I = ABC (resolution III). This quarter replicate did not allow the investigation of all two-way interactions since some were aliased with main effects. Hence this experiment is useful for screening when only the main effects and a limited number of two-way interactions are important. The information on interactions is a trade-off for running a smaller scale experiment. Therefore, though full experiments are not always necessary, care must be taken when selecting the fractional replicates first to avoid aliasing important factors, and secondly to prevent loss of information by neglecting the important interactions.

9.2.2 Three-levels and mixed-levels experiments

Experiment one (Section 6.4) was a screening experiment and this was followed by a confirmatory experiment in Section 6.5. In this experiment, there was the advantage of investigating an additional level for each selected factor. This is a verification experiment and with the remaining factors, there is the possibility to use a response surface to determine the changes in the response variables.

Experiments in Sections 7.2, 7.3 and 7.4 are mixed-levels experiments. Advantages of three-level and mixed-level experiments are:

(1) They were orthogonal arrays therefore, the analysis and interpretation of the results were more simplified.
(2) They obtained a lot of information about the main effects in a relatively few number of runs
(3) Non-linear terms can be investigated or included in the model especially in the three-levels experiments.
(4) In mixed-level experiments there are advantages of having different number of levels for different factors at one go.

The disadvantages of three-level and mixed-level experiments are:

1. They provide limited information about interactions
2. They can lead to a very big experiments and require more runs than a comparable 2^{k-p} design, and a two-level design will often suffice when the factors are continuous and monotonic.
3. The fractional designs of three-level and mixed-level experiments have more complex aliasing structure.

Therefore, in all factorial experimental designs, as the number of factors and levels increase, the experimental designs become more complicated and more difficult to analyse and interpret the data.

9.2.3 Comparison of factorial and Taguchi experiments

In this thesis, the novel application of Taguchi methodology was used for cryopreservation protocol development experiments. Taguchi methodology standardises the method for designing experiments using orthogonal arrays. This, in conjunction with the associated set of linear graphs which assign factors to the columns of the orthogonal array, allowed experiments to be designed rapidly to investigate the main effects and desired two-way interactions. The advantage of orthogonal arrays in designing experiments is its robustness in finding optimal treatment combination by reducing the number of treatment combinations to run compared to the full factorial experiment. For comparison purposes, full Taguchi experiments were conducted along with suitable fractional replicates. It was noted that fractionally replicated experiments analysed using SNRs provided similar conclusions as the full experiment and without sacrificing large number of seeds. This reduces the cost and time of the experiments and therefore has a great advance in cryopreservation protocol developments.

Identical significant main effects were noted using ANOVA and SNR method in all experiments except for shoot to root ratio response variable (Tables 6.8, 6.13, 7.5, 7.13 and 7.17). However, the Spearman's rank correlation between observed mean and SNR was highly significant in all the experiments showing that similar conclusions are obtained using either ANOVA or SNR methods.

However, some differences in determining the optimal treatment were found between ANOVA and SNR analyses of the same size experiment (e.g. shoot to root ratio and dry weight in Experiment 1 (Table 6.8), dry weight in Experiment 2 (Table 6.13) and dry weight in Experiment 3 (Table 7.5). Though the factorial experiments selected the treatment combinations with best means, they were not always the most robust as one or two outliers might influence the mean. The best treatment combinations suggested by using SNR are more likely to be distributed around the mean values). For these cases, the Taguchi choice provided a more robust solution.

Dot plots showed that the best treatments chosen using SNR are more consistent compared to the best treatments chosen using ANOVA. This suggests that similar or more informed conclusions could be obtained using SNR. Roy (1990), concluded that when the signal to noise ratio is used, the optimum condition identified from such analysis is more likely to produce consistent performance. Muthusamy *et al.,* (2005) showed that the same conclusions as factorial experiments could be obtained with Taguchi experiments. Furthermore, they claimed that a more consistent conclusion could be obtained using a Taguchi method as evidenced by the dot plots. These properties make Taguchi experimental design a favourable alternative to traditional designs.

Taguchi methodology uses signal to noise ratio for data analysis. The signal to noise ratio metric provides a robust mechanism to find the optimal treatment combination. This is when the SNRs are calculated following the desired categories (smaller-is-better, larger-is-better or nominal-is-better), the largest SNR is corresponding to the optimum treatment. The advantage of using a signal to noise ratio is it can be specifically designed to meet certain experimental criteria. Different SNRs are suitable for different types of response variables. For example, in the first experiment (Section 6.4) the shoot to root ratio response variable is desired to be a target number (one). Hence, 'nominal is best' criterion was used to calculate this SNR. This is an advantage of using SNR as variable compared to ANOVA in factorial experiments which only predicts the significance of the effects.

In practice, only fractionally replicated Taguchi experiments are conducted. One disadvantage of this in cryopreservation or any biological experiment is that prior knowledge about the system is essential before designing the experiments. This is especially true when considering what information is to be obtained from the experiments. For example, the prior information on which interaction will have effect on the response variable and which interaction can be ignored is a prerequisite before designing the experiment.

9.3 Cryopreservation

9.3.1 Orthodox seed cryopreservation

Successful cryopreservation of orthodox seeds is largely dependent on reducing the impact of two injurious effects; dehydration and imbibitional. Ice formation during cooling is limited if seeds are dried to very low moisture contents. This phenomenon can be cryo-protective as it reduces the amount of freezable water in the cells. However, if the dehydration effect is too great, cell damage occurs as a result of the reduced cellular water content (Mazur, 2004). Ultra-rapid cooling can be applied if cells are sufficiently dehydrated so that they become vitrified or ice crystals formed are so small that they do not cause injury. In Chapter 6, *C. siamea* seeds have been desiccated to moisture contents of 14%, below which all the osmotically active water content may have been removed. Therefore, ice-induced freezing injury does not occur and a high survival rate after cryopreservation is achieved. Both experiments on orthodox seed showed that as drying time increased (from 4 h to 8 hr), sprouting day was delayed and the seedlings had a larger shoot to root ratio (mainly due to shorter root development) perhaps due to imbibitional injury. Imbibitional damage is another problem when hydrating seeds desiccated to very low moisture contents (Bewley *et al.*, 2000). Gu and Xu (1985) suggested that there was some sensitivity to imbibitional damage that was completely reversible by slow re-hydration and that the degree of the damage was not sufficient to reduce viability. Jeller *et al.*, (2003) reported that mean sprouting day for *Cassia excelsa* seeds were delayed when water availability was smaller due to slow imbibition rate.

9.3.2 Intermediate seed cryopreservation

Seeds and embryonic axes were compared for the cryopreservation of an intermediate seed, (*K. malaccensis*). It was noted that seeds germinated more rapidly compared to embryonic axes after cryopreservation. Cryostorage, generally necessitates the use of excised zygotic embryonic axes that are sufficiently small to be rapidly partially dehydrated (Berjak *et al.*, 1999). However, other important contributing factor is the maturity stage of

the embryos. In practice the axis must be sufficiently mature to facilitate its ongoing development *in vitro*, which may necessitate a compromise between the optimal explant sizes for cryopreservation and subsequent plant development (Goveia *et al.*, 2004). Whilst orthodox seeds undertake maturation drying which programmes the switch from a developmental mode to a germinative mode (Kermode and Finch-Savage, 2002; Soeda *et al.*, 2005; Greggains *et al.*, 2000), many recalcitrant seeds especially in the tropics, do not undergo maturation drying and therefore, do not experience reduced cellular metabolism and a clear physiological termination of seed development (Pammenter and Berjak, 1999).

In this thesis, the effect of seed developmental stage on cryopreservation was investigated for *S. cordata* embryos (intermediate seed) for the first time. Seed developmental stage three (SDS 3) when the seeds are fully matured and shed from mother trees was found to be optimum for cryopreservation of these embryos. The percentage germination continued to increase with seed maturation as the moisture content decrease and dry weight increase. Freezing tolerance also increase sharply at the later stage of seed development, this might be related to the increase of the late embryogenesis accumulated (LEA) proteins, which are supposedly involved in the maturation and desiccation tolerance acquisition (Peterbauer and Richter, 2001).

9.3.3 Recalcitrant seed cryopreservation

Cryopreservation protocol development for desiccation-sensitive germplasm remains a challenge especially for those species which produce recalcitrant seeds. The development of a successful cryopreservation protocol is challenged by the fact that recalcitrant seeds are large and are shed with high moisture content (Krishnapillay, 2000). Consequently, lethal freezing damage invariably occurs if hydrated seeds are exposed to liquid nitrogen, while conversely drying the seeds to a moisture content where ice does not form, usually leads to desiccation damage and loss of viability (Benson, 2004). These requirements result in the establishment of a

successful cryopreservation protocol to achieve harmless dehydration of recalcitrant seed germplasm with moisture contents that are sufficiently low to allow survival following exposure to critical cryogenic temperatures. Therefore, in this thesis, slightly different approaches compared to orthodox and intermediate seeds were considered to develop vitrification-based cryopreservation protocol cryopreservation protocol for *P. speciosa* a recalcitrant seed producing species. These were based on three aspects:

1. Using shoot-tips from germinated seeds and their *in vitro* derived micropropagated plantlets as an approach to overcome seed limitation problem.
2. The application of Differential Scanning Calorimetry to elucidate the critical points at which vitrification occurs in the shoot-tips such that cryoprotection is achieved.
3. Novel application of trehalose cryoprotectant in the preculture medium which has been highly related to survival of polar biota as alternative to sucrose.

In this thesis, application of shoot tips as the solution for seed limitation problem in tropical rainforest tree species for cryopreservation studies was considered. This involves germinating the seeds in *in vitro* tissue culture and using micropropagation of shoots on MS medium before excising shoot meristems for cryopreservation studies. The advantage of this approach was it supplied sufficient material for the cryopreservation experiment. However, since the shoot-tips were exposed to *in vitro* conditions, they were subjected to many stresses even before applying the cryoprotective treatments.

9.3.4 Ice-free vitrification

The urgency and growing demand for methodological improvement of recalcitrant seed cryopreservation has stimulated discussion and much debate in the literature regarding the relative merits of traditional freezing methods compared to ice-free vitrification approaches (Engelmann, 2004).

Prevention of freezing by vitrification means that water in a tissue remains a glassy-liquid during cooling without crystallization (Mazur, 2004). As cooling proceeds, however, the molecular motion in the liquid permeating the tissue decreases. Eventually, an 'arrested liquid' state known as a glass is achieved, this conversion of a liquid into a glass is called vitrification. A glass is a liquid that is too cold or viscous to flow. Vitrification procedures have been developed for apices, cell suspensions, embryonic tissues and somatic embryos of numerous species (Hor *et al.*, 2005; Cyr, 2000; Sakai *et al.*, 2002).

Preculture (Normah, 2000; Dumet *et al.*, 2000a), post thawing handling including re-growth medium (Lynch, 1999) and applying appropriate vitrification procedures are essential for successful ice-free vitrification. A high level of sugar was reported to be very important for improving the survival of cryopreserved cells or meristems (Niino, 2000). In Section 7.4 of this thesis, *S. cordata* embryos were precultured on sucrose medium before cryopreservation. Sucrose is the most widely used dehydrating agent in preculture medium. Dumet *et al.*, (2000a), showed that sucrose pre-treatment had effects on both the desiccation and cryopreservation of oil palm (*Elaeis guineensis*) embryos at relatively high water content (higher than 3 gH_2O/g dry weight). The results in Section 7.4, showed neither sucrose concentration nor preculture time had any detrimental effect on the embryos and this treatment was important to achieve high germination after cryopreservation.

The novel application of trehalose, a cryoprotectant, in the preculture medium, which is important for the survival of polar biota has been explored as an alternative to sucrose for *P. speciosa* shoot-tips cryopreservation. The shoot-tips were precultured on trehalose medium at three different concentrations (2.5%, 5% and 10%) for 3 different durations (1,2 and 3 days). Crowe and Crowe's (1992), studies of artificial membranes, reported that trehalose substitutes for water molecules in the membrane during dehydration and thus helps to maintain membrane integrity. Spectroscopic evidence of the interaction of trehalose and phospholipids indicated that trehalose may hydrogen bond to the hydration shell in the head group region of the phospholipids (Crowe *et al.*, 2004). In addition, Crowe *et al.*, (2004)

also showed that trehalose inhibits the induction of the gel phase by reducing water content, and spreading phospholipid monolayers, as indicated by DSC studies.

Application of the chemical-based vitrification method did not initially result in high levels of survival for the majority of cultured plant cells except for a limited number of species or cultivars (Uragami, 1991; Sakai *et al.*, 1991). These observations suggest that direct exposure of cultured cells to a highly concentrated vitrification solution may lead to harmful effects due to osmotic stress or chemical toxicity. Harmful effects of PVS2 treatments can be alleviated or eliminated by reducing the concentration of PVS2 as well as the time of exposure to the level necessary for the dehydration of the cells after which the cell suspension may become vitrified. Another approach for the successful vitrification of cultured plant cells is the gradual addition of PVS2 (20%, 65% and 100%) (Abdelnour and Engelmann, 2002). A glycerol based, less toxic vitrification solution designed as PVS3 (Sakai, 2004) is also recommended. For successful cryopreservation by complete vitrification it is necessary to control carefully the procedures for dehydration and cryoprotectant permeation and to prevent injury by chemical toxicity or excessive osmotic stress during dehydration.

Evidence for vitrification requires the use of physical procedures. One conventional method is to measure the latent heat released by the crystallization of ice during cooling and warming by DSC. DSC is a powerful tool used to measure physical thermal parameters (freezing, melting and glass transitions) critical for the survival of cryopreserved tissues (Benson *et al.*, 1996b). In cryopreservation, freezing damage of cells is generally attributed to freeze-induced cell dehydration which results in a higher solutes concentration, reduced cell volume, and loss of structural (bound or osmotically inactive) water (Block, 2003). Of these processes, structural water has been suggested to be of major importance for maintaining membrane integrity. In Chapter 8, DSC thermal analysis was carried out on *P. speciosa* shoot-tips after various trehalose and PVS2 treatments. *P. speciosa* shoot-tips treated with PVS2 at different times were cooled to –150°C and then warmed at 10°C min^{-1}. For shoot-tips treated with PVS2 (30, 60 and 90 min), no freezing exotherm occurred during cooling (Figures

7.5, 7.6 and 7.7). However, during subsequent rewarming a series of changes in the thermal behaviour of the vitreous solid such as glass transition (60 min PVS2), exothermic devitrification (30 min PVS2) and endothermic melting (30 min PVS2) was observed. A more stable profile was obtained as the PVS2 treatment was increased to 90 min. These results indicate that the shoot-tips that were sufficiently dehydrated with PVS2 especially after 60 and 90 min and they became vitrified during rapid cooling. DSC has very accurately measured the nature and magnitude of thermal events during cooling-warming cycles, where an increase in survival is systematically correlated with a decrease in the intensity of ice crystallisation/melting events and optimal survival was reached when only vitrification events were recorded.

Problems of de-vitrification are mostly avoided in dry seeds where glass transition temperatures (Tg) are greater than the melting temperature (Tm) of water (Buitink *et al.*, 1996; Leprince and Walters, 1995). Deterioration rates of seeds correlate with intracellular viscosity (Buitink *et al.*, 2000; Walters *et al.*, 2004). Decreases in viability of cryogenically stored material have been attributed to non-intentional warming (most likely due to moving of cryopreserved vials in and out of cryotanks), resulting in an increase in the rate of damaging intracellular ice formation, which deteriorates the cells (Kim *et al.*, 2005; Sherlock *et al.*, 2005). Ice formation during warming is potentially as injurious to the cells as the cooling cycle. Devitrification can be avoided experimentally by using moderate warming rates. The rewarming of vitrified materials requires careful selection of heating rates sufficient to prevent significant thermal cracking, devitrification and recrystallization during warming. The use of carefully designed warming protocols is therefore necessary to maximise viability and maintain structural integrity. Vitrified materials, which may contain appreciable thermal stresses developed during cooling, may require an initial slow warming step to relieve residual thermal stresses. Despite developments to devise solutions that would vitrify at particular temperatures regimes for biological tissues, the corresponding critical warming rate necessary to avoid devitrification remains a critical challenge.

To conclude, cryopreservation by vitrification offers distinct advantages compared with procedures that allow or require ice formation. First, it does not require a controlled freezing equipment or sophisticated, expensive apparatus. The encapsulation-vitrification technique for cryopreservation which has recently been applied to a wide range of cultured meristems appears to be suitable method for the cryopreservation of *P. speciosa* shoot tips. Secondly, complete vitrification eliminates concerns for the known damaging effects of intra-cellular ice crystallization (Benson *et al.*, 2004). Thirdly, tissues cryopreserved by vitrification are exposed to less concentrated solutions of cryoprotectants for shorter time periods (Sakai, 2004). Finally, unlike conventional cryopreservation procedures that employ freezing, vitrification, do not require controlled cooling and warming at optimum rates (Engelmann, 2004). A principle benefit of vitrification is the elimination of prerequisite studies to determine optimal cooling rates for tissues with multiple cell types. Successful vitrification requires that the thermal processing be rapid enough through the glass transition regions of such that maximal ice crystal nucleation and growth that occur above the glass transition temperature of the solution is minimised and obviated completely. Thus, it is only necessary to cool solutions at rates in which a negligible fraction of the solution forms ice. Vitrified materials have a similar rate requirement during heating, when samples are re-warmed for subsequent use, to limit ice formation to negligible levels.

9.4 Conclusions

1. *C. siamea* species was successfully cryopreserved for the first time using a desiccation protocol and the best treatment combination was 9hrs desiccation, slow-rapid warming and germinated in filter paper medium which gave the highest viability of 90% (Table 6.8).

2. Taguchi experimental design using SNR was successfully applied to the cryopreservation experiments providing similar results compared to traditional factorial designs with almost the same conclusions for both factorial and Taguchi methods (Tables 6.8, 6.13, 7.5, 7.13 and 7.17).

4. Where slightly different conclusions were obtained using these two methods (shoot to root ratio and dry weight), more robust and consistent results were obtained using the Taguchi design.

5. Fractionally replicated experiments (both factorial and Taguchi) analyses concluded that full experiments are rarely necessary and fractional replicates of full factorial experiments are sufficient provided a priori identified two-way interactions can be tested for significance.

6. The correct conclusions for experiments 5 (Section 7.4) and 6 (Chapter 6) were assisted by use of appropriate data analysis (binary logistic regression analysis)

7. Both *K. malaccensis* seeds and embryos were successfully cryopreserved for the first time using a desiccation technique with high survival (above 80%) after cryopreservation. This study showed that cryopreservation could be a suitable alternative method for the long-term storage and conservation of *K. malaccensis* germplasm.

8. For *S. cordata* seeds, germination after cryopreservation was only affected by seed development stage, where embryos at SDS 3 showed greater germination. A successful cryopreservation protocol using

PVS2 vitrification was developed for *S. cordata* embryos with high germination (above 80%) after cryopreservation.

9. For *P. speciosa* shoot-tips, a cryopreservation protocol was developed using encapsulation-vitrification method after preculture in a trehalose medium. PVS2 treatment for 60 min was the optimal with stable glass formation during cooling and warming as evidenced by the DSC thermal analysis. This study exemplifies how DSC could be applied as an investigative tool to help formulate new cryopreservation strategies especially for problematic tropical recalcitrant seed with complicated physiology that has never before been cryopreserved.

9.5 Scope for Further Work

Having completed this thesis, there are areas discussed and described as follows where future work can be carried out in developing cryopreservation protocols for difficult germplasm:

1. Prediction of flowering and fruiting seasons in tropical rainforests using 'time series analysis' on phenological data will assist in germplasm acquisition for conservation and forest management programmes and link to the physiological status of seeds.

2. The use of Taguchi experiments in cryopreservation protocol development of other recalcitrant tropical rainforest tree species.

3. The use of Taguchi methodology for thermal analysis studies (e.g. DSC) for which cryoprotection treatments required to be developed rapidly within the constrain of using small samples.

4. The use of other available experimental designs apart from factorial and Taguchi for cryopreservation experiments. These include, Plackett and Burman (PB) designs which describe the construction of very economical designs with the run number of a multiple of four (rather than a power of 2) (Plackett and Burman, 1946). Plackett-Burman designs are very efficient screening designs when only main effects are of interest and are used for screening experiments because, in this design, main effects are, in general, heavily aliased with two-way interactions. The PB design in 12 runs, for example, may be used for an experiment containing up to 11 factors. PB designs also exist for 20-run, 24-run, and 28-run (and higher) designs. With a n-run design it is possible to run a screening experiment for up to (n-1) factors.

5. A broader application of the Taguchi methodology in the development of future conservation strategies for tropical rainforest germplasm. Especially for genetic resources from flora and fauna that are rare, at risk and endangered.

6. 'Hierarchical ordering principle' was used in this thesis and all three and higher way interactions were ignored. A rigorous investigation of whether this principle is reasonable in cryopreservation experiments could be investigated.

7. In Chapter 8, PVS2 components (glycerol, DMSO, ethylene glycol and sucrose) were not analysed individually using DSC. Therefore, the individual effects of these components on the thermogram were not detected. It will be useful to repeat the DSC analysis for these components to produce base line information. Therefore, the thermograms obtained with samples and PVS2 solution can be directly compared and the effects of each composite component on glass stability determined.

8. The presence of lipid reserves in seed tissues appears to be another key factor involved in survival of seeds after exposure to ultra-low temperature. Hor *et al.*, (2005) reported that for *Citrus* species studied, unfrozen water content of the seeds was negatively correlated to seed lipid content. Therefore, an investigation of the influence of the amount and composition of reserve lipids on the sensitivity of seeds to LN exposure in tropical forest tree germplasm is recommended.

9. Investigations of the functions of stress proteins in cryopreserved materials will aid the development of cryopreservation protocols. Especially having acquired the knowledge that different developmental stage poses different cryotolerance level in intermediate seed in Chapter 7. Stress proteins related to dehydrins, found in both higher (Close, 1997) and lower (Li *et al.*, 1997) plants. These proteins were first described in relation to dehydration resistance (Velten and Oliver, 2001; Volaire *et al.*, 2001), but subsequently they have been shown to be produced in response to chilling (Ismail *et al.*, 1999), freezing (Zhu *et al.*, 2000), wounding (Richard *et al.*, 2000), osmotic stress (Tabaei-Aghdaei *et al.*, 2000), and chemical injuries (Crowe *et al.*, 2001). Genes for several

dehydrins have been cloned (e.g., Svensson *et al.*, 2000), thus presenting opportunities to study functions of these proteins in dehydration stress especially in recalcitrant seeds, both *in vivo* and *in vitro*.

10. The application of trehalose in preculture medium is relatively rare compared to sucrose. However, recent studies on trehalose by Sakurai and Inoue (2004) showed new aspects of protection functions of this sugar in the preservation if biomaterials. Emphasising the antioxidant function of trehalose and the physicochemical origin of the cross-protection effect of trehalose against various stresses, including water and oxidant stresses. This aspect could be further investigated for tropical rainforest germplasm cryopreservation studies.

11. Investigation on free radical damage and antioxidant defence to oxidative stress during cryopreservation of tropical plant germplasm will provide more information and understanding for developing cryo-protocols especially for recalcitrant seeds. The complex morphology and physiology of recalcitrant seeds influence their responses to stresses and injury during cryopreservation. Monitoring the antioxidant status of plants during cryopreservation and their component parts would provide an understanding of the role of free radicals and oxidative mechanisms in normal physiological processes as well as in the study of deleterious oxidative stress.

12. The development of microscopic techniques to visualise events occurring during cryopreservation would provide further valuable insights into the mechanism of cryoinjury and recovery especially for recalcitrant germplasm.

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APPENDICES

List of Appendices

APPENDIX 1

INTRODUCTION TO FACTORIAL EXPERIMENTS

A1.1 Introduction

The importance of the rapid and efficient optimisation of cryopreservation protocols for tropical tree seed germplasm was highlighted in Chapter two. The design of experiments that reduces the amount of germplasm required to be sacrificed and rapidly screen cryogenic protocols would be an advantage. This section reviews techniques for designing and analysing traditionally used full and fractional factorial experiments at two, three and mixed-levels. Section A1.3. introduces the terminologies used in factorial experiments. Section A1.4. reviews the design of full factorial experiments at two levels, estimating main and interaction effects and using ANOVA to identify significant factors and interactions. Since small-scale experiments are favoured for tropical seed cryopreservation, emphasis is also given in this chapter on designing and analysing fractional factorial experiments.

A1.2 Experimental Designs

A design of an experiment is a plan for conducting it. In an experiment, purposeful changes are made to independent variables or factors for which the resulting behaviour of the response variable is observed (Box *et al.*, 1978). The experimental units or materials are assigned to different groups at random, which then are given different treatments. The groups are then compared on some response variable to determine the effects of the treatments. Experiments are performed mainly for two purposes; first to identify important factors affecting the response variables and secondly, to optimise the response variables (Wu and Hamada, 2000).

A1.3 Basic Experimental Components

This section defines the terminologies used throughout the thesis for factorial experiments.

1. **Experimental units** are the objects, which receive treatments. These are the units where the response is measured. In this study, tropical forest tree seeds, embryos and shoot tips were used as experimental units.
2. **A response variable** is the response or measurement carried out on the experimental units which provides the data for statistical analysis. Examples of response variables selected for this study were, sprouting day, shoot to root ratio and seedling dry weight.
3. **Factors** are independent variables controlled by the experimenter. In this study, many factors were chosen in cryopreservation experiments, for example, desiccation time, desiccation method, cooling and warming rates.
4. **Treatment levels** are the different values chosen for a factor. For example in this study, desiccation time was varied for two and four hour in the first experiment.
5. **Treatment Combination** also known as a run is the combination of the settings of several factors in a given experimental trial. For example, there are 32 runs in an experiment with five factors at two levels each (2^5).
6. **Replication** is the number of times a specific treatment combination is repeated during an experiment.

7. **The number of observations** is the number of the treatment combinations multiplied by the number of replications. For example, in an experiment with five factors at two levels each with three replications, there are 3 x 32 = 96 observations.
8. **Design of the experiment** is the plan for conducting the experiment, including what factors are used and how the factors are set. The design also translates into a plan for the statistical analysis of the experiment. Three different designs of experiments were compared in this study; these are the full factorial (Section A1.4), fractional factorial (Section A1.5) and Taguchi experiments (Chapter 4).
9. **Full Factorial Design** is a design that combines the levels for each factor with all the levels for every other factor.
10. **Fractional Factorial Design** is a design that contains a fraction of the full factorial experiment.

There are many reasons for undertaking factorial experiments. First, it is applied in exploratory work for the quick determination of main effects (Section A1.4.2) of factors. In the initial phases of an investigation, factorial experiments can establish which factors significantly affect the response variable (Wu and Hamada, 2000). Secondly, factorial approaches are used to investigate interaction effects (Section A1.4.3) between all combinations of the factors (Box *et al.*, 1978). The third application of factorial experiments is in tests undertaken to lead to recommendations that must apply over a wide range of conditions (Wu and Hamada, 2000).

A1.4 Full Factorial Experiments At Two-Levels

In the full factorial approach, the investigator compares all treatments that can be formed by combining the levels of the different factors (Box *et al.*, 1978). It is highly efficient, because every observation supplies information about the method of investigating the relationship between the effects of different factors. Usually the treatments are assigned randomly to the pool of the experimental units with an equal number of units in each treatment.

An experiment with all possible high/low combinations of all the input factors is called a full two-level factorial design. If there are k factors, each at two levels, a full factorial design has 2^k runs. Therefore, a 2^3 experiment can be defined as an experiment with three factors at two levels each, hence with (2 x 2 x 2) eight treatment combinations or runs. The general way to describe a single level factorial experiment is q^k, where q is number of levels of each factor and k is the number of factors.

A common experimental design is one with all input factors set at two levels each. These levels are commonly called `high' and `low' of the each factor (Montgomery, 2001). There are many notations used to code these two levels in the factorial experiments. The first notation is '0' and '1', which represent the two levels respectively. Table A1.1 shows a full factorial experiment with three factors at two levels with '0' and '1' notation. The defining contrast if found by using each factor to the power of the row. For example, notation 1 0 0 in run two in Table A1.1 becomes $A^1B^0C^0 = A$ and hence the first run is $A^0B^0C^0$ or 1.

Table A1.1 Full factorial experiment with three factors at two-levels

Run	Defining contrast	A	B	C
1	1	0	0	0
2	A	1	0	0
3	B	0	1	0
4	AB	1	1	0
5	C	0	0	1
6	AC	1	0	1
7	BC	0	1	1
8	ABC	1	1	1

The second type of notation in the factorial experiment uses '-1' and '+1' for the two levels. In this notation, the defining contrast is the factor with '+1' in that run. For example, the same experiment in Table A1.1 can be written as in Table A1.2. This type of notation is useful when calculating main effects and interactions effects as described in Sections A1.4.2 and A1.4.3.

Table A1.2 General layout for a full factorial experiment with three factors at two-levels

Run		Defining contrast	I	A	B	C
1	1	(no factors at level '+1')	+1	-1	-1	-1
2	A	(factor A at level '+1')	+1	+1	-1	-1
3	B	(factor B at level '+1')	+1	-1	+1	-1
4	AB	(factors AB at level '+1')	+1	+1	+1	-1
5	C	(factor C at level '+1')	+1	-1	-1	+1
6	AC	(factors AC at level '+1')	+1	+1	-1	+1
7	BC	(factors BC at level '+1')	+1	-1	+1	+1
8		ABC (factors ABC at level '+1')	+1	+1	+1	+1

The column 'I' is an identity element in the group of multiplication (Weisstain, 2005). This is an element with a neutral action. When the identity element is combined with any element of the group in the group operation the result is always to give back the same member of the group. For multiplication of real number of the identity element is one; for addition of real number is zero. For example, when A is multiplied with I: A x I = I x A = A. The third notation used for experiment with two-levels are '1' and '2' which is more commonly used in Taguchi experiments (refer Chapter 4).

A1.4.1 Example of full factorial experiment at two-levels

An example of a full factorial experiment with three factors at two levels (coded as ± 1) is given below. A cryopreservation experiment is conducted where seed biomass was assessed after storage in liquid nitrogen after one of two desiccation times with one of two different cooling and two warming rates.

The factors and levels are:

A: desiccation time (-1 = 2hr, 1 = 4hr)

B: cooling rate (-1 = rapid cooling, 1 = slow cooling)

C: warming rate (-1 = rapid warming, 1 = slow warming)

The coding (–1 and 1) for factor A represent numbers and for factor B and C represent categories. Note also that as in all full factorial experiments, every combination of the factor levels has been used (Table A1.3).

Table A1.3 Full factorial experimental layout for the example experiment

Run	Defining contrast	A (Desiccation time)	B (Cooling rate)	C (Warming rate)	Biomass (g)
1	1	-1	-1	-1	58 = Y_1
2	A	1	-1	-1	69 = Y_2
3	B	-1	1	-1	51 = Y_3
4	AB	1	1	-1	65 = Y_4
5	C	-1	-1	1	50 = Y_5
6	AC	1	-1	1	81 = Y_6
7	BC	-1	1	1	45 = Y_7
8	ABC	1	1	1	78 = Y_8

The outcomes of the above experiment could also be represented by the following diagram. The cube plot below illustrates how the biomass values change when the factors change from –1 to +1 level. For example, when B and C are at level –1, the biomass value when A is –1 level is 58 (as indicated in Figure A1.1) and it increases to 69 when A changes to level +1.

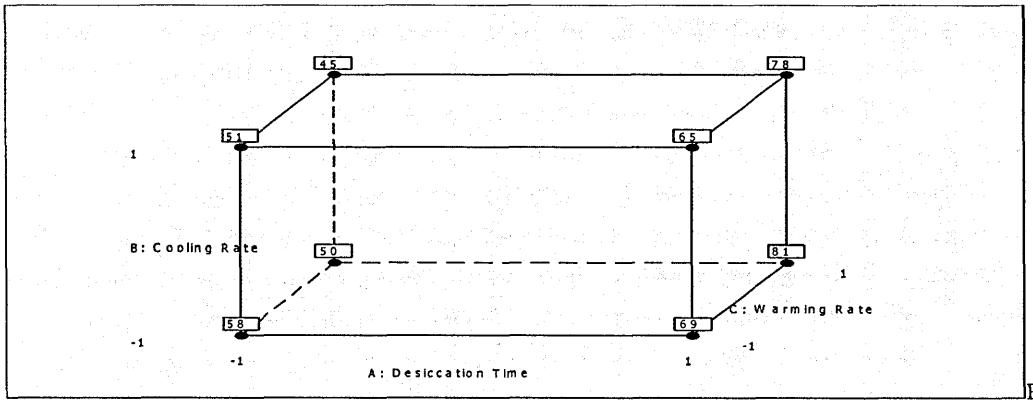

Figure A1.1 Cube plot for seed biomass

A1.4.2 Estimating main effects

"A main effect is the direct effect of an independent factor on the response variable" (Box *et al.*, 1978). The main effect of A (varying desiccation time from level -1 to level +1 or from 2 to 4hr) can now be estimated. There are four pairs of observations where factor A (desiccation time) changes but the other factors are equal. For example, the main effect of factor A can be estimated by:

from y_2-y_1 = 69-58 = 11
from y_4-y_3 = 65-51 = 14
from y_6-y_5 = 81-50 = 31
from y_8-y_7 = 78-45 = 33

The main effect of A is defined as the average of these estimates.

$= {}^1/_4 \{ (y_2 - y_1) + (y_4 - y_3) + (y_6 - y_5) + (y_8 - y_7)\}$

$= \frac{1}{4} \{ (y_2 + y_4 + y_6 + y_8) - (y_1 + y_3 + y_5 + y_7)\}$

$= \frac{1}{4}\{ (69 + 65 + 81 + 78) - (58 + 51 + 50 + 45)\}$

$= \frac{1}{4} (293) - \frac{1}{4} (204) = 73.25 - 51 =$ **22.25**

Therefore, on average, other factors being equal, increasing desiccation time from 2 to 4 hours increases biomass by 22.25 units.

The general formula for estimating a main effect (e.g. for factor A) in a 2^k experiment is:

$$\frac{1}{r2^{k-1}}\left\{\sum Ay_+ - \sum Ay_-\right\}$$

Where, ΣAy_+ = sum of observations for all level +1 for factor A;
ΣAy_- = sum of observations for all level –1 for factor A;
k = number of factors;
r = is the number of replicates.

Therefore for main effect of B(cooling rate):

$$\frac{1}{r2^{k-1}}\left\{\sum By_+ - \sum By_-\right\}$$

since there is only one replicate, r= 1 and k=3 (for 3 factors)

= ¼ { (y_3 + y_4 + y_7 + y_8) - (y_1 + y_2 + y_5 +y_6)}
= ¼ {(51 + 65 + 45 + 78) – (58 + 69 + 50 + 81)}

= ¼ (239)- ¼ (258)

= 59.75 – 64.5 = **-4.75**

Therefore, on average, other factors being equal, changing cooling rate from rapid to slow decreases biomass by 4.75 units.

Similarly for main effect of C (warming rate):

$$\frac{1}{r2^{k-1}}\left\{\sum Cy_+ - \sum Cy_-\right\}$$

= ¼ { (y_5 + y_6 + y_7 +y_8)- (y_1 + y_2 + y_3 + y_4) }

= ¼ (50 + 81 + 45 +) – ¼ (7858 + 69 + 51 + 65)
= ¼ (254)- ¼ (243)
= 63.5 - 60.75 = **2.75**

Hence, on average, other factors being equal, changing warming rate from rapid to slow increases biomass by 2.75 units.

These main effect estimations are illustrated by the main effects plots produce by the statistical package Minitab® as shown in Figure A1.2. This shows that factors A has a larger effect than factors B and C. For factor A level 1, the estimated calculated value was 73.25 which is also marked in the graph. This is the mean value of four observations at level 1 for factor A, as shown in

the calculation. Similarly, for factor A level –1, the calculated value was 51 which is also shown in Figure A1.2. For factor B, the level 1 value was 59.75 and –1 value was 64.5 and for factor C, the level 1 value was 63.5 and –1 value was 60.75.

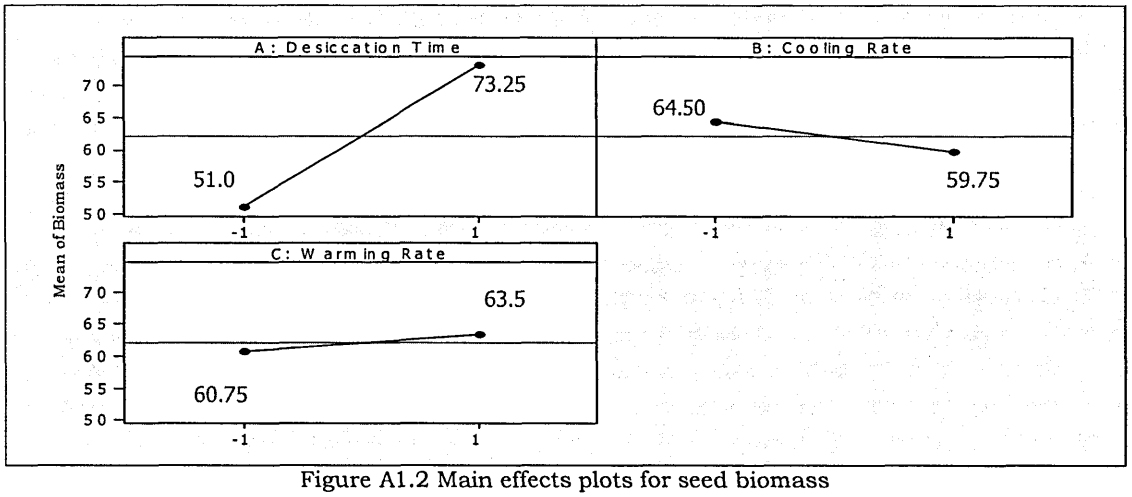

Figure A1.2 Main effects plots for seed biomass

A1.4.3 Estimating interaction effects

"An interaction effect is the joint effect of two or more independent factors on the response variable" (Box *et al.*, 1978). When considering the interaction between two factors, the term two-way interactions are used in this thesis.

AC two-way interaction

The average estimate of desiccation time (A) where warming rate (C) is at level 1 (slow) is, $\frac{1}{2}((y_2-y_1)+(y_4-y_3)) = \frac{1}{2}(11+(14)) = 12.5$

Similarly, the average estimate of desiccation time (A) with rapid warming rate observations is, $\frac{1}{2}((y_6-y_5)+(y_8-y_7)) = \frac{1}{2}(31+33) = 32$

The two-way interaction of factor A and C the AC interaction is defined as half of the difference. Therefore, the AC interaction = ½ (32-12.5) = **9.75**, which can also be calculated as follows:

$= \frac{1}{2}\{\frac{1}{2}[(y_6-y_5)+(y_8-y_7)] - \frac{1}{2}[(y_2-y_1)+(y_4-y_3)]\}$

$= \frac{1}{4}\{(y_1+y_3+y_6+y_8) - (y_2+y_4+y_5+y_7)\}$

$= \frac{1}{4}\{(58+51+81+78) - (69+65+50+45)\}$

$= \frac{1}{4}(268) - (229) =$ **9.75**

Referring back to the experimental design, the columns for interactions AB, AC, BC and ABC can be created by multiplying the levels of the relevant factors as shown in Table A1.4. For example, consider column AB run number Y_2 which has level –1. This level is be created by multiplying level A (+1) and B (-1) in the same row.

Table A1.4 Full factorial experimental layout with all interactions

Run	A	B	C	AB	AC	BC	ABC	Biomass (g)
Y_1	-1	-1	-1	1	1	1	-1	58
Y_2	1	-1	-1	-1	-1	1	1	69
Y_3	-1	1	-1	-1	1	-1	1	51
Y_4	1	1	-1	1	-1	-1	-1	65
Y_5	-1	-1	1	1	-1	-1	1	50
Y_6	1	-1	1	-1	1	-1	-1	81
Y_7	-1	1	1	-1	-1	1	-1	45
Y_8	1	1	1	1	1	1	1	78

The observations that are in the first round of bracket ($y_1 + y_3 + y_6 + y_8$) come from the AC column at level 1 and the second round of bracket ($y_2 + y_4 + y_5 + y_7$) come from the AC column at level –1. Similarly, considering the AB column in Table A1.4, The AB interaction can be calculated as follows;

AB Interaction = ¼ { ($y_1 + y_4 + y_5 + y_8$) - ($y_2 + y_3 + y_6 + y_7$)}

= ¼ (251) – (246) = **1.25**

The observations that are in the first round of bracket ($y_1 + y_4 + y_5 + y_8$) come from the AB interaction at level 1 and the second round of bracket ($y_2 + y_3 + y_6 + y_7$) come from the AB interaction at level –1.

Similarly, it can be shown that the BC interaction is:

= ¼ { ($y_1 + y_2 + y_7 + y_8$) - ($y_3 + y_4 + y_5 + y_6$)}

= ¼ (250) – (247) = **0.75**

Three and higher-way interactions can be found in the same way. Therefore the estimate of the ABC interaction is

½ ($y_2 + y_3 + y_5 + y_8$) – ½ ($y_1 + y_4 + y_6 + y_7$)

= ½ (69 + 51 + 50 + 78) – ½ (58 + 65 +81 + 45)

= ½ (248) – ½ (249)

= 124 – 124.5 = **-0.5**

As for main effects, statistical packages could be used to plot interaction effects. Figure A1.3 shows the Minitab output of the two-way interaction plot for factors A, B and C.

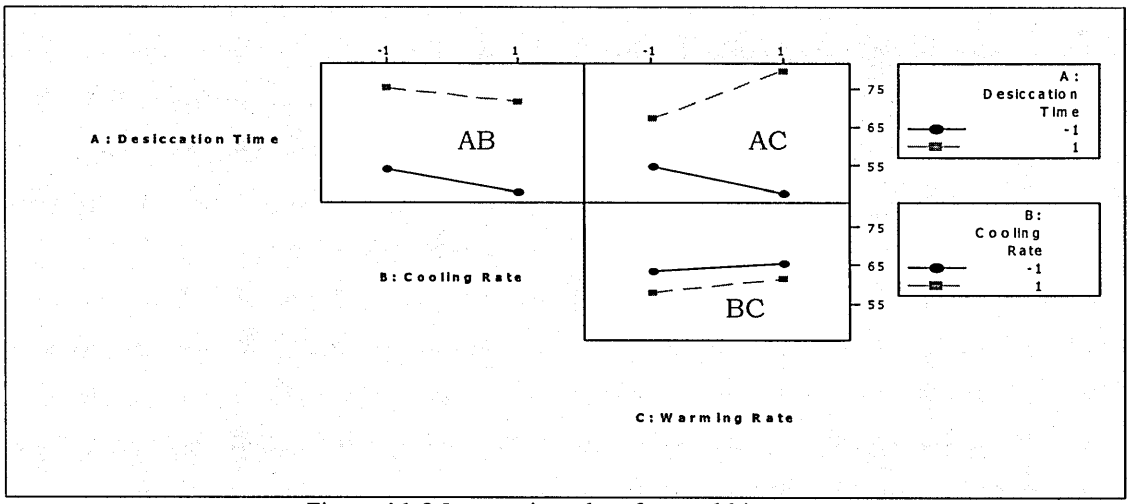

Figure A1.3 Interaction plots for seed biomass

When there is no interaction, the main effect of one factor does not change across levels of the other factor(s). Hence, the lines in the above plot are approximately parallel. This observation is noted for the AB and BC interactions. However, the main effect of factor A changes across levels of factors C and vice versa. This shows that there is an interaction between these two factors, which is also in agreement with the calculation where this interaction has the highest value of 9.75.

A1.4.4 Finding significant effects using Analysis of Variance

In the estimation of main and interaction effects, it was noted that the effects vary in size (e.g. effect of A = 22.25 and effect of B = -4.75). It is important to distinguish those factors and interactions with effects that are not equal to zero. Statistical inference could be used to determine which effects have a value significantly different from zero, i.e. a non-zero value that is not just due to chance. One method to do this is by carrying out an Analysis of Variance (ANOVA). The key statistic in ANOVA is the F-test of difference of group means, testing if the means of the groups formed by values of the factor (or combinations of values for multiple factors) are different enough not to have occurred by chance (Box, 1954). If the group means do not differ significantly then it is inferred that the factor(s) did not have an effect on the response variable (Winer *et al.*, 1991).

One-way ANOVA (also known as univariate ANOVA, simple ANOVA, single classification ANOVA, or one-factor ANOVA) tests for differences in a single response variable among two or more levels of a single factor (Iverson and Helmut, 1987). It can be extended to n-way ANOVA which analyses one response variable for n factors (Larry, 1993).

It should be noted that as the number of independent factors increases, the number of interactions is given by the binomial coefficient, $\frac{k!}{x!(k-x)!}$, where, k=total number of factors and k!= k factorial = 1x2x3x...k, x=number of letters in interactions (e.g. x=1 for main effects, x=2 for two-way interaction). For example, the number of six-way interaction in an experiment with ten factors is given by

$$\frac{10!}{6!(10-6)!} = \frac{10!}{6!4!} = \frac{10\times9\times8\times7\times6\times5\times4\times3\times2\times1}{(6\times5\times4\times3\times2\times1)(4\times3\times2\times1)} = \underline{\mathbf{210}}$$

Two factors have a single two-way interaction (AB). Three factors have three two-way interactions (AB, AC, BC) and one three-way interaction (ABC), or four in all (as described in Section A1.3.1.2). Four factors have six two-way (AB, AC, AD, BC, BC, CD), four three-way (ABC, ABD, ACD, BCD), and one four-way (ABCD) interaction, or eleven in all. As the number of interactions increase, it becomes increasingly difficult to interpret the results of the ANOVA (James, 1998).

The F-test, also called the F-ratio, is an overall test of the null hypothesis that the group means of the response variable do not differ. It is used to test the significance of each main and interaction effect. It is possible that the significant difference may be very small for large samples. Therefore, it is not only significance that is important, but also strength of association. If the F score is enough above one, it will be found to be significant in a table of F values, using (k – 1)

(number of groups minus one) degrees of freedom for between-groups and (n – k) (sample size minus the number of groups) for within-groups degrees of freedom. If F is significant, then it can be concluded that there are differences in the group means, indicating that the factors have an effect on the response variables. If the probability of F is less than 0.05 for any effect, it is concluded that that the effect does have an affect on the response variable (Thornton, 1988).

The ANOVA table has two parts of output. As an illustrative example, consider the experiment introduced in Section A1.4.1 with three factors (A, B and C) at two levels each now with two replications (Table A1.5). The two parts of ANOVA analysis produced by Minitab for this experiment are shown in Tables A1.6a and A1.6b.

Table A1.5 Full factorial experiment for three factors at two-levels with two replications

Run	A	B	C	Biomass (Replicate 1)	Biomass (Replicate 2)	Biomass (Mean)
Y_1	-1	-1	-1	58	52	55.0
Y_2	1	-1	-1	69	65	67.0
Y_3	-1	1	-1	51	53	52.0
Y_4	1	1	-1	65	60	62.5
Y_5	-1	-1	1	50	41	45.5
Y_6	1	-1	1	81	71	76.0
Y_7	-1	1	1	45	38	41.5
Y_8	1	1	1	78	61	69.5

Table A1.6a Estimated Effects and Coefficients for seed biomass

Term	Effect	Coef	SE Coef	T	P
Constant		58.625	1.531	38.29	0.000
A	20.250	10.125	1.531	6.61	0.000
B	-4.500	-2.250	1.531	-1.47	0.180
C	-1.000	-0.500	1.531	-0.33	0.752
A*B	-1.000	-0.500	1.531	-0.33	0.752
A*C	9.000	4.500	1.531	2.94	0.019
B*C	-0.750	-0.375	1.531	-0.24	0.813
A*B*C	-0.250	-0.125	1.531	-0.08	0.937

The model for above analysis is;

Biomass = 58.625 +10.125A –2.25B –0.5C –0.5AB +4.5AC –0.375BC –0.125ABC

For run one in Table A1.5 where all the factors have level –1, the fitted values for the biomass is = 58.625 +10.125(-1) –2.25(-1) –0.5(-1) –0.5(-1x-1) +4.5(-1x-1) –0.375(-1x-1) –0.125(-1x-1x-1) = **55.**

Therefore, the residual which is the difference between observed and fitted values for replicate 1 is = 58-55 = 3; and for replicate 2 = 52-55 = **-3**

Table A1.6a contains columns labeled

- **Term:** the main effects and interactions in the ANOVA model as showed above. Note that A*B represents the AB two-way interaction.
- **Effect:** the effect as found in Section A1.4.2 and A1.4.3 for a particular main effect or interaction. For example, the effect for the main effect of A is 20.25. This effect can be calculated using the same method as in Section A1.4.2 but using the mean response.

- **Coefficient**: is the gradient difference between level +1 and –1 for the factors and interactions. For 2^k experiments coded –1 and +1, the coefficient is divided by two since there are 2 units difference when an effect moves form -1 to +1.
- **Standard error coefficient (Se-Coefficient)**: is defined as the square root of adjusted mean square for residual error divided by the total number of runs (Montgomery, 1991). The mean squares for residual error is explained in the second part of ANOVA table output.
- **T-statistic**: is the coefficient value for each input divided by the standard error coefficient. For example, for Factor A the coefficient was 10.125 and the standard error coefficient was 1.531, therefore, 10.125 divided by 1.531 gives a T value of 6.61. If there were no effect, T follows a t-distribution with one degree of freedom (Murdoch and Barnes, 1986).
- **p-value:** is the component of the ANOVA table that serves as a measure of significance. If the number found in this column is less than the critical value set by the experimenter (usually 0.05), then the effect is defined as significant. Therefore, when the p-value is less than 0.05, it can be concluded that there is a significant relationship between two variables and any value greater than this value will be interpreted as a non-significant effect. In the above table, the significant effects were noted for main effect A and two-way interaction of AC while the main effects of factors B, C and all other interactions are not significant at the 5% significant level.

Table A1.6b Second part of Analysis of Variance for seed biomass

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Main Effects	3	1725.25	1725.25	575.083	15.34	0.001
2-Way Interactions	3	330.25	330.25	110.083	2.94	0.099
3-Way Interactions	1	0.25	0.25	0.250	0.01	0.937
Residual Error	8	300.00	300.00	37.500		
Total	15	2355.75				

- **Degrees of Freedom (df):** For an effect, the degree of freedom is the number of levels minus one. Since each factor and interaction in a 2^k experiment has two levels, each has (two minus one) one degree of freedom. Hence, the three main effects have a total of three degrees of freedom, the three two-way interactions have three degrees of freedom and the single three way interaction has one degree of freedom. The degree of freedom (df) for residual error is found by subtracting the df for the effects from the total df (15-3-3-1=8). The total degrees of freedom is equal to total number of observations minus one (16-1 =15 in above experiment).
- **Sums of Squares (Seq SS):** For an effect, the sums of squares is defined as sum of squares of the difference between the estimated value of an observation and the overall mean. Consider the main effect A: each of the eight values with +1 is estimated by their mean, 68.75 and each of eight values with –1 is estimated by their mean, 48.5. The overall mean of all 16 observations is 58.625. Therefore, the sum of squares for A= $16 \div 4\ (68.75 - 58.625)^2 + 16 \div 4\ (48.5 - 58.625)^2 = 4(10.125)^2 - 4(10.125)^2 = (20.25)^2 \times 4 = 1640.25$. In general for a 2^k experiment SS can be defined as; SS (treatment) = $(\text{effect})^2 \times$ number of observations/4). In this experiment the number of observations are 16, therefore, for main effect B: the sum of squares for B = $(-4.5)^2 \times 4 = 81.0$. Similarly for main effect C: $(-1.0)^2 \times 4$

= 4.0. Hence, the sums of squares for main effects A, B and C is = 1725.25 (Table A1.6b). The SS for interaction terms are found similarly. The residual errors are found by subtracting the seq SS for all main effects and interaction form the total seq SS. Therefore, for above experiment, it is 2355.75 – 1725.25-330.25-0.25 = 300.

- **Adjusted sum of squares (Adj SS)**= Seq SS
- **Adjusted MS (Adj MS)** = Adj SS divided by degrees of freedom. For example for main effects, adjusted MS = 1725.25/3 = 575.08 (as shown in Table A1.6b).
- **F-value=** Adj MS divided by Adj MS residual. For example, for main effects, F = 575.08 / 37.5 = 15.34
- **P-value =** This is the probability of falsely rejecting the null hypothesis. The p-value in this part of ANOVA table are used to determine which of the effects (main effects or interaction effects) in the model are significant. If there are interactions in the analysis, the effect of higher order interaction need to be considered first since they have influence on the main effects in the ANOVA model.

In this thesis, the ANOVA results are reported as the F or t and p-values for relevant effects. Therefore, for above example, the main effect of A has a significant effect on biomass [t(8)=6.61, p=0.000]. The main effects are significant [F(3,8)=15.34, p=0.001]. The p-values are summarised into three categories. A single '*' indicates significant at 5% significance level, '**' indicates significant at 1% significance level, and a '***' indicates significant at 0.1% significance level.

A1.4.4.1 ANOVA assumptions

There are three assumptions in ANOVA (Larry, 1993):

1. The groups formed by the factors are relatively equal in size
2. Within each group, the response variable data follow a normal distribution.
3. The variance (the square of the standard deviation) of the normal distribution is the same for each group (homogeneity of variance).

A1.4.4.2 Groups are equal in size

The first assumption is that the groups formed by the factors are relatively equal in size. This assumption is true when there are equal number of observations for each factor. In this thesis, all the experiments were designed with an equal number of replicates. Although there were some missing values, all the experiments have approximately an equal number of observations.

A1.4.4.3 Testing normality

Normal distribution of residuals is an assumption of ANOVA (Winer *et al.*, 1991). Residuals are differences between the observed values and the corresponding values that are predicted by the ANOVA model (Thornton, 1988). The normal distribution of a response variable could be checked by performing a normality test on its residual. For example, for experiment in Table A1.4, the normality check is carried out using the Anderson-Darling test (Stephens, 1974) on the biomass residual produced by ANOVA. Figure A1.4 shows the probability plot for this analysis which shows that the data are normally distributed since the p-value is 0.678 which is greater than 0.05. If the data are perfectly normal, then the data points on the probability plot will form a straight line.

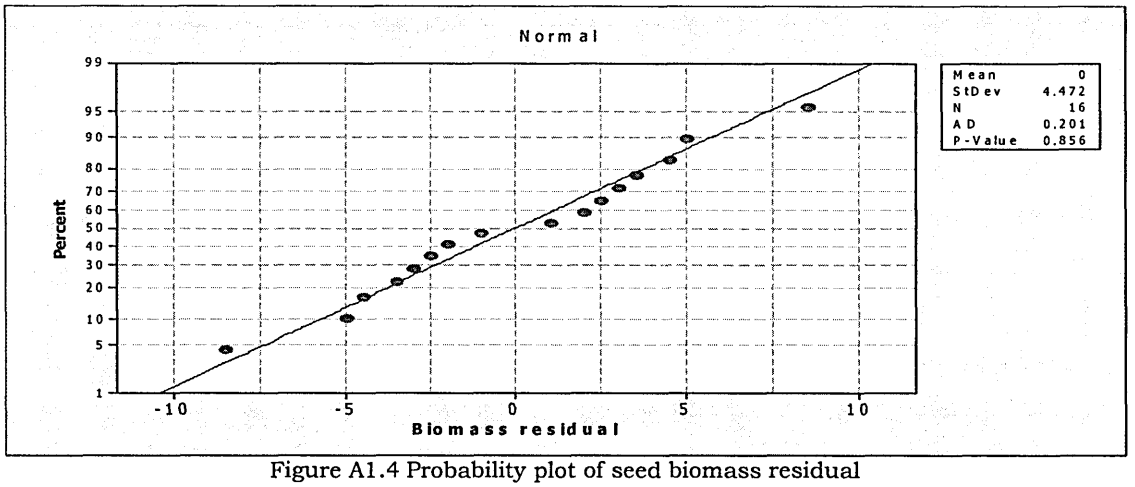

Figure A1.4 Probability plot of seed biomass residual

A1.4.4.4 Homogeneity of variance

The third assumption of ANOVA is the response variable should have the same variance in each category of the independent variable (Winer *et al.*, 1991). When there is more than one independent variable or factor, there must be homogeneity of variances in the cells formed by each combination of the independent variables. The reason for this assumption is that the denominator of the F-ratio is the within-group mean square, which is the average of group variances taking group sizes into account. When groups differ widely in variances, this average is a poor summary measure. However, ANOVA is robust for small and even moderate departures from homogeneity of variance assumption (Box, 1954).

Bartlett's test of homogeneity of variance is computed by Minitab can be used to test the ANOVA assumption that each group (category) of the factors have the same variance. If the Bartlett statistic is significant at the 0.05 level, the null hypothesis that the groups have equal variances is rejected. And if the significant level is above 0.05, then the null hypothesis that the groups have equal variances is accepted. For the above experiment, the Minitab output of Bartlett's test shows a p-value of 0.824. Therefore, it is concluded that the groups of the factors have equal variances.

In all the experiments in this thesis, the diagnostic checks for both normality and homogeneity of variance were carried out to ensure the conclusions derived from ANOVA are valid.

A1.4.5 Disadvantages of full factorial experiments

A full factorial experiment provides a lot more information than a single factor experiment, with potentially not much more work (Montgomery, 1991). However, one limitation of the full factorial experiment is that the number of the treatment combinations rises rapidly with the number of factors (Staines *et al.*, 1999) as illustrated in Table A1.7. For example, a full factorial experiment with seven two-level factors would require 128 treatment combinations to investigate all main effects and interactions. This large number of samples can also be highly labour and cost intensive. This could be a problem in a cryopreservation protocol development experiment with tropical seeds, where experiments need to be conducted rapidly using scarce resources. A more economical approach is to use a carefully selected fraction of the full factorial design that allows the main effects and two-way interaction effects to be estimated.

Table A1.7 Number of runs for a 2^k full factorial experiment

Number of Factors (k)	Number of Runs
2	4
3	8
4	16
5	32
6	64
7	128

A1.5 Fractional Factorial Experiment At Two-levels

A fractional factorial design is a carefully chosen subset of the full factorial experiment (Wu and Hamada, 2000). The notation used to define the experiment is similar to the full factorial experiment. The single level fractional factorial experiment can be written as q^{k-p}, where q is number of levels, k = total number of factors and p is the fraction of the experiment. For example, 2^{5-1} denotes, an experiment with one half of the full five two-level factors and hence has 16 runs.

Fractional factorial design is a procedure to reduce the number of observations required for a full factorial design. For example, a full design for five factors, each at two levels, results in 2^5 or 32 combinations, whereas ½ or ¼ fractional design requires only 16 or eight treatments respectively (Babiak, 1998). It is especially suitable for screening or preliminary experiments, in which the objective is to determine the (hopefully) few experimental factors that have a significant effect on the response variable. However, the reduction in data collected comes with a price, since not all interactions can be estimated, though in many cases estimating higher order interaction is of dubious value. According to the 'hierarchical ordering principle', main effects tend to dominate two-way interactions, and these in turn dominate higher-way interaction effects. This suggests that three and higher order interactions can be routinely ignored (Wu and Hamada, 2000).

A1.5.1 Aliasing in fractional factorial experiments

Selection of the particular fraction to be included in the experiment, which will allow the investigation of desired main effects and/or interactions must be made with considerable care. There are two main criteria to select a fractional replicate of a full factorial experiment.

The first criterion is 'minimising aliasing effects' (Montgomery, 2001). Two factors are aliased when their effects cannot be estimated separately or when two factors are set at the same levels throughout the experiments (Anderson and Whitcomb, 1997). For example, consider an experiment with three factors, A, B and C at two-levels each. A full factorial experiment will have eight runs as shown in Table A1.8.

Table A1.8 Full factorial experiment with three factors at two-levels

Run	Defining contrast	A	B	C	AB	AC	BC	ABC
1	I	-1	-1	-1	1	1	1	-1
2	A	1	-1	-1	-1	-1	1	1
3	B	-1	1	-1	-1	1	-1	1
4	AB	1	1	-1	1	-1	-1	-1
5	C	-1	-1	1	1	-1	-1	1
6	AC	1	-1	1	-1	1	-1	-1
7	BC	-1	1	1	-1	-1	1	-1
8	ABC	1	1	1	1	1	1	1

A half fractional replicate of the above experiment will have four runs. If the full design can be reduced to a half fraction by suppressing all runs with -1 level for ABC interaction the experimental design will be as in Table A1.9.

Table A1.9 Half fractional experiment for an experiment with three factors at two-levels

Run	Defining contrast	A	B	C	AB	AC	BC	ABC
2	A	1	-1	-1	-1	-1	1	1
3	B	-1	1	-1	-1	1	-1	1
5	C	-1	-1	1	1	-1	-1	1
8	ABC	1	1	1	1	1	1	1

Notice that the column for the factor A, is identical to the column for the BC interaction. This experimental design cannot separate these two effects (main effect A and two-way interaction BC). The analysis results of this reduced design will have information for either factor A or the BC interaction but not both. These two are said to be aliased and written A=BC. Therefore, if this fractional replicate were to be used for this experiment, a decision needs to be made whether factor A or the BC interaction is regarded as important. Usually main effects are given priority according to the 'hierarchical ordering principle' (Wu and Hamada, 2000). Note that:

1. In general for 2^{k-p} experiments a half experiment mean that pairs of effects are aliased. If a quarter of the experiment is performed four effects are aliased together, etc.
2. The pattern of aliasing depends on which subset is selected. If, for example, those observations with a –1 in the ABC were selected then A=-BC. If those observations with a 1 in the column BC were selected then B would be aliased with C and A with ABC.

To determine which effects are aliased together for particular subsets selected modular arithmetic is used. An example of modular 12 is a clock face, where for 13:00 hours, the clock face will show 1 o'clock, and written as 13= 1(modulus 12). For 15:00 hours it will show 3 o'clock and it is written as 15=3 (modulus 12).

For experiments with two levels, modular two is used. Note that for each main effect or interaction column using the +/- 1 notation, there square = 1 (since $1^2 = (-1)^2 = 1$). In general, $A^x = A^y$, where y=x (modulus 2) is equal to 0 or 1 (this is not the case for 0,1 notation) and hence A x A = 1, A^3 = A x A x A = A^2 x A = 1 x A = A, where generally powers follow modular arithmetic.

The aliasing relationship for the above experiment is denoted as;

I = ABC (since the runs used in the experiment have one in the ABC column). This is known as a defining relation (Franklin, 1985).

When both sides are multiplied with A,

I * A = BC or A = BC i.e. A is aliased with BC as shown above. Since multiplying I with A will give A. Similarly, B= B * I = B x (ABC) = A x B^2 x C = AC (since $B^2 = B^0 = 1$). Hence B is aliased with the Ac interactions as shown in Table A1.9.

A1.5.2 Resolution

The second criterion for the selection of a fractional factorial design is 'resolution' (Montgomery, 2001). Resolution describes the degree to which estimated main effects are aliased with estimated two-way interactions, three-way interactions, etc. In general, the resolution of a design is number of letters in the defining contrast (Franklin, 1985).

For example, in the above experiment, I = ABC, this is known as resolution III (since there are three letters in the defining contrast). Most references on fractional factorial design use Roman numerals to denote the resolution of a design (Chen *et al.*, 1993). A common notation for a q^{k-p} with resolution r is q^{k-p}_r. For example, 2^{5-1}_V denotes a half (2^{-1}) design for five two-level factors (A to E) with resolution V (i.e. has designing contrast I=ABCDE). The implication of the resolution on the alias of main effects and two-way interactions is shown below:

In resolution V

- No main effects are aliased with any other main effects
- No main effects are aliased with any two-way interaction
- No two-way interactions are aliased with any two-way interactions
- Two-way interactions are aliased with three-way interactions

In Resolution IV

- No main effects are aliased with any other main effects
- No main effects are aliased with any two-way interaction
- Some two-way interactions are aliased with other two-way interactions

Therefore this resolution is sufficient if only main effects and some two-way interactions are of interest.

In Resolution III

- No main effects are aliased with any other main effects
- At least one main effect is aliased with a two-way interactions
- At least one two-way interaction is aliased with a three-way interaction

Therefore this design is only useful to investigate main effects when not all of the two-way interactions are regarded as important since it is not possible to separate all the main effects with all the two-way interactions.

The choice of the resolution to design a fractional experiment depends on the aims of the experiment (the information needed to be obtained), material, cost and time availability. It also should be noted that it is not always possible to have higher resolutions for smaller fractional

experiments. Table A1.10 shows the resolutions and design generators which could be used to construct a required fractional factorial experiment. For an experiment with three factors, the highest possible resolution is resolution III to construct a half fractional replicate which is shown in Table A1.9.

Table A1.10 Resolutions and design generators for constructing some fractional factorial experiments (Adapted from Chen *et al.*, 1993)

Number of factors	Number of runs in full experiment	Fraction	Number of runs in fractional experiment	Resolution	Defining relation
3	8	½	4	III	I=ABC
4	16	½	8	IV	I=ABCD
5	32	½	16	V	I=ABCDE
		¼	8	III	I=ACE

A1.5.3 Construction of a half fractional factorial experiment with five factors at two-levels

An example on how a half fractional factorial experiment can be designed from a full factorial experiment with five factors at two levels each is shown below. Consider a cryopreservation experiment with five factors (A, B, C, D and E) each at two levels (Table A1.11):

A: desiccation time (-1= 2hr, +1= 4hr)
B: cooling rate (-1= rapid, +1= slow)
C: LN exposure [-1=-LN, +1=+LN]
D: warming rate (-1= rapid, +1 = slow)
E: BAP hormone level in recovery media (-1=1 mgl^{-1}; +1= $2mgl^{-1}$)

For the above experiment, out of 63 (number of observations ($(2 \times 2^5) - 1 = 64$) degrees of freedom, five will be used to find the main effects, 10 for two-way interactions, 10 for three-way interactions, four for four-way interactions and one for five-way interaction. Hence, fifteen degrees of freedom are allocated for three or higher-way interactions. Since three or more way interactions are regarded as not important, this is not a very efficient design. A half fractional of above experiment can be designed with only 16 runs instead of 32 runs which would allow all main effects and two-way interactions to be estimated. Therefore, this would reduce the number of seeds required to be sacrificed in that experiment without losing important information. There are three choices of resolutions (V, IV and III) and many design generators to create a half fractional factorial experiment for the above experiment as shown in three examples below;

Example 1: Resolution V

The design generator for this resolution is I = ABCDE. This has resolution V since there are five characters in the defining contrast. The alias structure for A can be found by multiplying both sides with A

$A = A \times I = A \times ABCDE$

$= (A^2) \times BCDE = (I) \times BCDE$ *($A \times A = A^2 = A^0 = 1$ according to modulus 2)*

$= BCDE$

This shows that factor A is aliased with the interaction BCDE. Hence for the runs in this experiment, the column for factor A is identical to the column for the BCDE interaction. Consequently this experimental design cannot separate these two effects. Similarly, the alias structure for all main factors and interactions could be identify as shown below:

1) I=ABCDE		I x B = ABCDE x B		B= ACDE
2) I=ABCDE		I x C = ABCDE x C		C = ABDE
3) I=ABCDE		I x D = ABCDE x D		D = ABCE
4) I=ABCDE		I x E = ABCDE x E		E = ABCD
5) I=ABCDE		I x AB = ABCDE x AB		AB = CDE
6) I=ABCDE		I x AC = ABCDE x AC		AC = BDE
7) I=ABCDE	→	I x AD = ABCDE x AD	→	AD = BCE
8) I=ABCDE		I x AE = ABCDE x AE		AE = BCD
9) I=ABCDE		I x BC = ABCDE x BC		BC = ADE
10) I=ABCDE		I x BD = ABCDE x BD		BD = ACE
11) I=ABCDE		I x BE = ABCDE x BE		BE = ACD
12) I=ABCDE		I x CD = ABCDE x CD		CD = ABE
13) I=ABCDE		I x CE = ABCDE x CE		CE = ABD
14) I=ABCDE		I x DE = ABCDE x DE		DE = ABC

This design is sufficient to investigate all main effects and two-way interactions. In this resolution (V), no main effects are aliased with any other main effects or any two-way interaction. In addition, no two-way interactions are aliased with any two-way interactions but two-way interactions are aliased with three-way interactions (e.g. AB and CDE).

Example 2: Resolution IV

Design generator: I = ABCD

Using the same approach, the alias structure for main effects and two-way interactions is:

A = BCD
B = ACD
C = ABD
D = ABC
E = ABCDE
AB = CD
AC = BD
AD = BC
BE = ACDE
AE = BCDE
CE = ABDE
DE = ABCE

In this resolution (IV), no main effects are aliased with any other main effects or two-way interaction. Some two-way interactions are aliased with other two-way interactions. Therefore this resolution is sufficient if we are interested to investigate only the main effects and some two-way interactions (BE, AE, CE and DE but not AB, CD, AC, BD AD and BC).

Example 3: Resolution III

Design generator: I=ABC

The alias structure for main effects and two-way interactions is:

A = BC
B = AC
C = AB
D = ABCD
E = ABCE
AD = BCD
AE = BCE
BD = ACD

BE = ACE
CD = ABD
CE = ABE
DE = ABCDE

In this resolution (III), no main effects are aliased with any other main effects but some are aliased with two-way interactions. Other two-way interactions are aliased with three-way interactions.

For the above experiment, the half fractional factorial experiment could constructed using resolution V (design generator I=ABCDE). The full experimental design is shown in Table A1.11. The runs with the effect ABCDE equal to -1 (highlighted) or those with ABCDE equal to +1 could be used for the half replicate. The particular half used (ABCDE = -1 or +1) is chosen at random. Assuming that the 16 observations with ABCDE = -1 is chosen, the design used is shown in Table A1.12.

Table A1.11 Full-factorial design of experiment for five factors at two levels

Run	Defining contrast	A	B	C	D	E	ABCDE
1	**I**	**-1**	**-1**	**-1**	**-1**	**-1**	**-1**
2	A	1	-1	-1	-1	-1	1
3	B	-1	1	-1	-1	-1	1
4	**AB**	**1**	**1**	**-1**	**-1**	**-1**	**-1**
5	C	-1	-1	1	-1	-1	1
6	**AC**	**1**	**-1**	**1**	**-1**	**-1**	**-1**
7	**BC**	**-1**	**1**	**1**	**-1**	**-1**	**-1**
8	ABC	1	1	1	-1	-1	1
9	D	-1	-1	-1	1	-1	1
10	**AD**	**1**	**-1**	**-1**	**1**	**-1**	**-1**
11	**BD**	**-1**	**1**	**-1**	**1**	**-1**	**-1**
12	ABD	1	1	-1	1	-1	1
13	**CD**	**-1**	**-1**	**1**	**1**	**-1**	**-1**
14	ACD	1	-1	1	1	-1	1
15	BCD	-1	1	1	1	-1	1
16	**ABCD**	**1**	**1**	**1**	**1**	**-1**	**-1**
17	E	-1	-1	-1	-1	1	1
18	**AE**	**1**	**-1**	**-1**	**-1**	**1**	**-1**
19	**BE**	**-1**	**1**	**-1**	**-1**	**1**	**-1**
20	ABE	1	1	-1	-1	1	1
21	**CE**	**-1**	**-1**	**1**	**-1**	**1**	**-1**
22	ACE	1	-1	1	-1	1	1
23	BCE	-1	1	1	-1	1	1
24	**ABCE**	**1**	**1**	**1**	**-1**	**1**	**-1**
25	**DE**	**-1**	**-1**	**-1**	**1**	**1**	**-1**
26	ADE	1	-1	-1	1	1	1
27	BDE	-1	1	-1	1	1	1
28	**ABDE**	**1**	**1**	**-1**	**1**	**1**	**-1**
29	CDE	-1	-1	1	1	1	1
30	**ACDE**	**1**	**-1**	**1**	**1**	**1**	**-1**
31	**BCDE**	**-1**	**1**	**1**	**1**	**1**	**-1**
32	ABCDE	1	1	1	1	1	1

Table A1.12 Half fractionally replicated experimental design

Run	Defining contrast	A	B	C	D	E
1	1	-1	-1	-1	-1	-1
2	AB	1	1	-1	-1	-1
3	AC	1	-1	1	-1	-1
4	BC	-1	1	1	-1	-1
5	AD	1	-1	-1	1	-1
6	BD	-1	1	-1	1	-1
7	CD	-1	-1	1	1	-1
8	ABCD	1	1	1	1	-1
9	AE	1	-1	-1	-1	1
10	BE	-1	1	-1	-1	1
11	CE	-1	-1	1	-1	1
12	ABCE	1	1	1	-1	1
13	DE	-1	-1	-1	1	1
14	ABDE	1	1	-1	1	1
15	ACDE	1	-1	1	1	1
16	BCDE	-1	1	1	1	1

A1.5.4 Analysing fractional factorial experiment at two-levels

As in the full factorial experiment, main and interaction effects are estimated using the methods described in Sections A1.4.2. and A1.4.3. The experiment can also be analysed using ANOVA as described in Section A1.4.4. For example, for the experiment in Table A1.5, a half fractional experiment is designed using defining relation I=ABC with resolution III as shown in Table A1.13.

Table A1.13 Half fractional experiment for an experiment with three factors at two-levels

Run	Defining contrast	A	B	C	Biomass Replicate 1	Biomass Replicate 2
1	A	1	-1	-1	69	65
2	B	-1	1	-1	51	53
3	C	-1	-1	1	50	41
4	ABC	1	1	1	78	61

The ANOVA output for the above half fractional experiment is shown in Table A1.14.

Table A1.14 ANOVA results for half fractional experiment

Term	Effect	Coef	SE Coef	T	P
Constant		58.500	2.469	23.70	0.000
A	19.500	9.750	2.469	3.95	0.017
B	4.500	2.250	2.469	0.91	0.414
C	-2.000	-1.000	2.469	-0.41	0.706

R-Sq = 80.58%

Analysis of Variance for Biomass (coded units)

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Main Effects	3	809.00	809.000	269.67	5.53	0.066
Residual Error	4	195.00	195.000	48.75		
Total	7	1004.00				

The above analysis shows that only factor A is significant ($t(4) = 3.95$; $p < 0.05$). Note that only the main effects could be investigated due to the alias structure and limited degrees of freedom. The fractional replicate experiments could also be used to predict the response variable for the other fractional replicates which was not conducted. This is undertaken by carrying out a stepwise regression analysis on the response variable from the fractionally replicated experiment which is used to predict the unobserved response variables for the full experiment (Refer to Section A2.2).

A1.6 Factorial Experiment At Three-levels

In some experiments, factors that have more than two levels are useful. For example, the addition of a third level allows the potential quadratic relationship between the factors and the response variable to be modelled. The three-level design with k factors, each with three levels is written as a 3^k factorial design (Montgomery, 2001). The three levels of the factors could be as low, intermediate and high and designated by the digit 0 (low), 1 (intermediate), and 2 (high) or as –1 (low), 0 (intermediate) and +1 (high).

Each treatment combination in the 3^k design will be denoted by k digits, where the first digit indicates the level of factor A, the second digit indicates the level of factor B, and the k^{th} digit indicates the level of factor K. For example, in a 3^2 design, 00 denotes the treatment combination corresponding to A and B both at the low levels (using the first notation), and 01 denoted the treatment combination corresponding to the A at the low level and B at the intermediate level (using the second notation).

A1.6.1 Example of a 3^3 design

Consider an experiment with three factors (A, B and C) each at three levels. This is a 3^3 factorial design, with experimental layout and treatment combinations as shown in Table A1.15.

Table A1.15 Experimental design for a full factorial 3^3 experiment

Run	A	B	C
1	-1	-1	-1
2	0	-1	-1
3	1	-1	-1
4	-1	0	-1
5	0	0	-1
6	1	0	-1
7	-1	1	-1
8	0	1	-1
9	1	1	-1
10	-1	-1	0
11	0	-1	0
12	1	-1	0
13	-1	0	0
14	0	0	0
15	1	0	0
16	-1	1	0
17	0	1	0
18	1	1	0
19	-1	-1	1
20	0	-1	1
21	1	-1	1
22	-1	0	1
23	0	0	1
24	1	0	1
25	-1	1	1
26	0	1	1
27	1	1	1

The 27 treatment combinations have a total of (27-1) 26 degrees of freedom. Each main effect has (number of levels minus one = 3-1) two degrees of freedom, each two-way interaction has (2 x 2) four degrees of freedom, and the three-factor interaction has (2 x 2 x 2) eight degrees of freedom. The main effects and interactions could be estimated as follows:

- Main-effects: If the main effects are quantitative and equally spaced, then the main effects could be portioned into linear and quadratic components each with one degree of freedom (Montgomery, 2001).. The linear effect can be interpreted as the difference between the average response at the low and high settings of the respective factors. The estimate for the quadratic effect can be interpreted as the difference between the average response at the centre (intermediate) setting and the combined high and low settings for the respective factors. If the main effects are qualitative, the effect of intermediate and high are compared to the low level.

- Interaction effects: The two-way interaction could be decomposed into linear x linear (AB), linear x quadratic (AB^2), quadratic x linear (A^2B), and quadratic x quadratic (A^2B^2) effects. The linear x linear interaction can be interpreted as half the difference between the linear main effect of one factor at the high and low settings of another. Similarly, the interactions of the quadratic components can be interpreted as half of another; that is either the high or low setting (for quadratic x linear interaction), or the intermediate or high and low settings combined (for quadratic x quadratic interaction).

In a 3^3 factorial experiment, (3-1) degrees of freedom will be associated with one factor. Therefore:

- Main effects will have two degrees of freedom
- Two-way interactions (e.g. AB interaction can be partitioned into two terms AB^2 and AB each with two degrees of freedom)
- Three-way interaction will have eight degrees of freedom. (e.g. ABC can be partitioned into four components: AB^2C^2, AB^2C, ABC^2, ABC).

As for the 2^k design, ANOVA could be used to identify significant effects in a 3^k design. However, unlike for the 2^k design for each main effect and interaction, one can test for the linear effect and the quadratic effect. In practice, the quadratic interactions are usually avoided due to difficulty in the interpretability of results. For example, a quadratic x quadratic interaction for factors A and B, indicates that the non-linear effect of factor A will change in a non-linear term with the setting of factor B. This means that there is complex interaction between these two factors present in the data that will make it difficult to understand and optimise the whole experiment.

One main disadvantage of a 3^k factorial experimental design is the size of experiment increases rapidly with k. For example, a 3^3 design has 27 treatment combination, a 3^4 design has 81 and a 3^5 design has 243. Hence, the concept of fractional replication can be extended to the 3^k factorial designs. However, some of the fractional replicates have a very complex alias structure.

A1.6.2 Designing fractional factorial experiments at three-levels

The general mechanism of generating fractional design at three levels is very similar to that of fractional factorial two level designs (Wu and Hamada, 2000). The largest fraction of the 3^k design is a one-third fractional, the 3^{k-1} fractional factorial design. Each main effect or interaction estimated from 3^{k-1} design has two aliases which are determined by multiplying the effect by both I and I^2

modulus three. To design a fractional replicate, first, the full factorial design need to be generated. As an illustrative example, consider a three-factor three-level factorial experiment, which has 27 treatment combinations. Assume that a $^1/_3$ replication is to be designed to obtain a 3^{3-1} design.

Any components out of the four ABC interactions above (generally, the highest-order interaction) could be selected to construct a fractional design. Each component will produce three replicates and therefore, there are 12 different one-third fractions of the 3^3 design as defined by

$$x_1 + \alpha_2 x_2 + \alpha_3 x_3 = L \text{ (modulus 3)}$$

where, α_i = 1 or 2 and L = 0, 1 or 2 and modulus 3 stands for modulus 3 arithmetic and any multiples of 3 equals to zero, multiples of 3-2 equal to 1, multiples of 3-1 equal to 2.

If the $I = AB^2C^2$ alias structure is selected, each fraction of the resulting 3^{3-1} design will contain exactly $3^2 = 9$ treatment combinations which should satisfy

$$x_1 + 2x_2 + 2x_3 = L(\text{modulus } 3)$$

Two rules are followed when selecting these alias structures:

1. First letter should have power of one
2. The group formed by F and F^2 are the same

Therefore, when a first letter does not have power of one, then the whole structure is squared since this would have the same value according to rule number two (Wu and Hamada, 2000).

The first alias of A is obtained by multiplying both side of $I = AB^2C^2$ with A;
$A \times A = A \times (AB^2C^2) = A^2B^2C^2 = ABC$ *(first letter should have power of one and group formed by F and F^2 are the same)*
Similarly, the second alias of A
$= A \times (AB^2C^2)^2 = A^3 \times (B^3 \times B) \times (C^3 \times C) = I \times (I \times B) \times (I \times C) = BC$ *(Modulus 3)*

The aliases of B $= B(AB^2C^2) = AB^3C^2 = AC^2$ *(Modulus 3)*
and $B(AB^2C^2)^2 = A^2B^5C^4 = A^2B^2C^1 = (A^2B^2C^1)^2 = ABC^2$ *(first letter should have power of one and group formed by F and F^2 are the same)*

The aliases of C $=C(AB^2C^2)=AB^2C^3=AB^2$ *(Modulus 3)*
and $C(AB^2C^2)^2 = A^2B^4C^5 = (A^2BC^2)^2 = AB^2C$

The Aliases of AB $=AB(AB^2C^2) = A^2B^3C^2 = (A^2C^2)^2 = AC$ *(first letter should have power of one and group formed by F and F^2 are the same)*
and $AB(AB^2C^2)^2 = A^3B^5C^4 = B^2C = BC^2$ *(first letter should have power of one and group formed by F and F^2 are the same)*

Since the main effects are aliased with two-way interactions, this is a resolution III design. The alias relationships are very complex compared to 2^k experiments, where each main effects is aliased with a component of the interaction. If for example, the two-way interaction BC is large, this will potentially misrepresent the estimation of main effect A.

Fractional replicates could be designed for $I = AB^2C^2$ equal to $L=x_1+2x_2+x_3$ modulus three. In a treatment combination, (0,0,0) represents factors A, B and C at levels 0 or ($x_1=0$, $x_2=0$, $x_3=0$),

therefore, if this is substituted in $L=x_1+2x_2+2x_3$ modulus 3, L= 0 +2(0) + 2(0) = 0, which corresponds to the second row of the third column, L value in Table A1.16. This is repeated until all the $L=x_1+2x_2+2x_3$ mod_3 values are calculated and treatment combinations of same L (L = 0, 1 or 2) values are grouped together in three replicates (Table A1.16).

Table A1.16 Treatment combination for factors A, B and C at three-levels with same L values grouped together

Replicate	Treatment Combination	L	Treatment Combination	L	Treatment Combination	L
1	000	0	101	0	202	0
	012	0	110	0	211	0
	021	0	122	0	220	0
2	001	1	102	1	200	1
	010	1	111	1	212	1
	022	1	120	1	221	1
3	002	2	100	2	201	2
	011	2	112	2	210	2
	020	2	121	2	222	2

A1.6.3 Analysing factorial experiments with three-levels

The main effects and interaction effects can be estimated for three-level experiments as described in Sections A1.4.2. and A1.4.3. and ANOVA could be used to analyse this type of experiment. However, the three-level experiments have the advantage of estimating the quadratic effects of the factors investigated. Main effect plots could be use to investigate the potential quadratic effects of the factors and the response surface methodology (Chandrasena *et al.*, 1997) could be used to investigate the possible quadratic effects of the factors (Refer Section 4.3.6)

A1.7 Mixed-level Factorial Experiments

There are many experiments especially biological experiments that require the consideration of factors with a different number of levels. The useful characteristics of two-level fractional factorial experiments, such as resolution and aliasing can be extended to the experiments involving multi-level factors (Ankenman, 1999). Wu and Zhang (1993), also presented various methods for designing and analysing experiments with mixed-levels.

A1.7.1 Construction of a mixed-level factorial experiment

Montgomery, (1991) suggested how to add three-level factors starting with two-level designs to obtain a mixed-level design. This process is divided into two steps. The first step involves generating a design for one factor, A at two levels and another factor, X at three levels from a 2^2 design with factors B and C both at two levels. The BC interaction is converted to factor X, with low

level when both B and C are at -1 levels; medium level when B and C are at combined (-1 or +1) levels; and high level when both B and C are at level +1 (Table A1.17).

Table A1.17 Creating a mixed-level design-first step

Run	Two Level			Three Level
	B	**C**	**BC**	**X**
	-1	-1	+1	X_1 (low)
	+1	-1	-1	X_2 (medium)
	-1	+1	-1	X_2 (medium)
	+1	+1	+1	X_3 (high)

Factor X has two degrees of freedom, which can be broken out into a linear and a quadratic component as in a 3^k design. In the next step, a experiment with three factors (A, B and C) at two levels each need to be designed (Table A1.18)

Table A1.18 Generation of a mixed level design with one factor at two-levels and another at three-levels from a 2^3 design

Run	**A**	**B**	**C**	**AB**	**AC**	**BC**	**ABC**	**A**	**X**	
1	-1	-1	-1	+1	+1	+1	-1	Low	Low	(B & C both low)
2	+1	-1	-1	-1	-1	+1	+1	High	Low	(B & C both low)
3	-1	+1	-1	-1	+1	-1	+1	Low	Medium	(B & C different)
4	+1	+1	-1	+1	-1	-1	-1	High	Medium	(B & C different)
5	-1	-1	+1	+1	-1	-1	+1	Low	Medium	(B & C different)
6	+1	-1	+1	-1	+1	-1	-1	High	Medium	(B & C different)
7	-1	+1	+1	-1	-1	+1	-1	Low	High	(B & C both high)
8	+1	+1	+1	+1	+1	+1	+1	High	High	(B & C both high)

In this example assume that the levels for factor X is created by referring to the BC interactions as shown in Table A1.17. Therefore, a new design is created with factor A at two levels (low and high) and factor X at three levels (low, medium and high). If the quadratic effect is negligible, a second two-level factor, D could be included by substituting D=ABC. Using the same method, a two- and four-level mixed design could also be created from a 2^4 (16 runs) design. AB or AC interactions could be used to create the four levels of factor X. In this example, as shown in Table A1.19, the AB interaction was used to create levels for factor X. Therefore, finally there will be factors C and D at two-levels and factor X at four levels.

Table A1.19 A single-four-level factor and two-level

Run	**(A**	**B)**	**=X**	**C**	**D**
1	-1	-1	X_1	-1	-1
2	+1	-1	X_2	-1	-1
3	-1	+1	X_3	-1	-1
4	+1	+1	X_4	-1	-1
5	-1	-1	X_1	+1	-1
6	+1	-1	X_2	+1	-1
7	-1	+1	X_3	+1	-1
8	+1	+1	X_4	+1	-1
9	-1	-1	X_1	-1	+1
10	+1	-1	X_2	-1	+1
11	-1	+1	X_3	-1	+1
12	+1	+1	X_4	-1	+1
12	-1	-1	X_1	+1	+1
14	+1	-1	X_2	+1	+1
15	-1	+1	X_3	+1	+1
16	+1	+1	X_4	+1	+1

A1.7.2 Analysing mixed-level experiments

The main effects and interaction effects can be estimated for mixed-level experiments as described in Sections A1.4.2. and A1.4.3. and ANOVA could be used to analyse this type of experiments. In this thesis, mixed-level experiments were used for cryopreservation of tropical intermediate (Chapter 7) and recalcitrant (Chapter 8) species. This is because, there were many parameters to be investigated and technically it could not be divided into a few small, different experiments since the seed availability for the species studied was limited and the fruiting season unpredictable.

A1.8 Summary

This chapter reviewed, in detail, designing and analysing full and fractional factorial experiments at two, three and mixed-levels. Full factorial experiments at two-levels are the simplest experiments but the disadvantage with this design is that the number of treatment combination increases rapidly with the number of factors. Fractional factorial experiments have a smaller number of treatment combinations but they need to be selected with care to avoid aliasing important effects.

Three-level experiments have the advantage of investigating quadratic effects but as with the two-level factorial experiments, their size increase exponently with the number of factors. However, fractional designs of three-level experiments have more complex aliasing structure.

Mixed-level factorial experiments have the advantage of investigating many factors at one go, but they have same disadvantages as the three level experiments with regards to experimental size and complex aliasing.

Therefore, in all factorial experimental designs, as the number of factors and levels increase the experimental designs become more complicated and more difficult to analyse and interpret the data. It is therefore an advantage to have a standard method to design and analyse experiments. The next chapter will review one such approach, the Taguchi technique for designing and analysing experiments.

APPENDIX 2

REGRESSION ANALYSES

A2.1 Introduction

Chapters three and four reviewed factorial and Taguchi experiments and their analyses using ANOVA. Selection of an analysis for an experiment depends on the design of experiment, choice of number of factors and their levels and types of response variables. This is particularly important in the design of cryo-conservation experiments for the preservation of at-risk, endangered, recalcitrant and difficult to obtain rainforest tree germplasm. Carefully designed experiments followed by stringent tools of statistical analysis that aids the fast throughput of technical procedures with the least sacrifice of important germplasm must be paramount. This chapter therefore, reviews the different regression and correlation analyses used in this thesis.

A2.2 Regression Analysis

A2.2.1 Simple linear regression

Simple linear regression is used to predict values of one variable, given values of another variable (e.g. if a seedling's height is to be predicted from its seed weight) (Draper and Smith, 1998). Consider a sample of ten seeds for which their dry weights and their seedlings heights are known. Assume that the values are plotted on a scatter diagram, with seed weight on the x-axis and seedling height on the y-axis. If there were a perfect linear relationship between seed weight and seedling height, then all 10 points on the graph would fit on a straight line. If there were a (nonperfect) positive linear relationship between seed weight and seedling height, then there would be a cluster of points on the graph which slopes upward. In other words, seeds with greater weight should give seedlings with greater height. If a linear relationship is on a scatter diagram, the best model can be found from a straight line. The simple linear regression equation is of the form

$$y_i = \beta_o + \beta_1 X_i + \epsilon_i$$

where,

y_i = i^{th} observation of the response variable, y,

i = 1, 2, 3..n, where n is the number of observations

X = i^{th} observation of the predictor variable, X

β_1= a constant (the gradient of the straight line)

$$\hat{\beta}_i = \frac{n\sum xy - \sum x \sum y}{n\sum x^2 - \left(\sum x\right)^2}$$, where n=number of observations

β_0 = a constant, the Y-axis intercept

$$\hat{\beta}_0 = \frac{\sum y - \left(\sum x\right)\beta_1}{n}$$

ϵ_i = i^{th} independently and identically distributed error term, IID $N(0, \sigma^2)$

Example: Consider an experiment for seed weight and seedling height for 10 randomly sampled seeds (Table A2.1).

Table A2.1 Data set for seed weight and seedling height

Seed weight (g)	Seedling height (cm)
3.3	16
7.1	18
13.2	34
10.2	36
9.3	30
6.4	19
4.2	15
5.1	13
12.2	38
11.6	30

A scatter diagram for above data is shown in Figure A2.1. A fitted regression line plot for the data is shown in Figure A2.2.

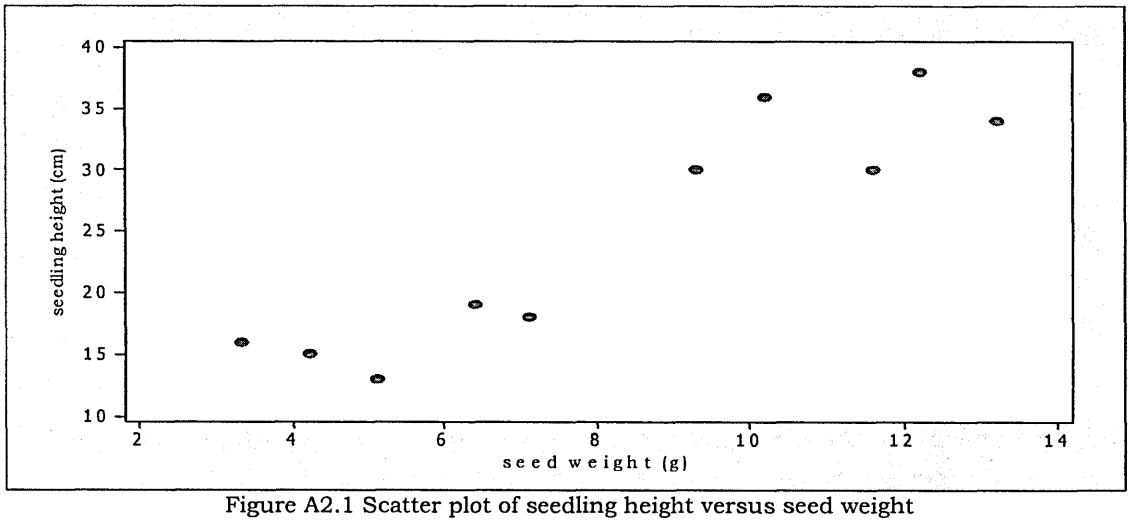

Figure A2.1 Scatter plot of seedling height versus seed weight

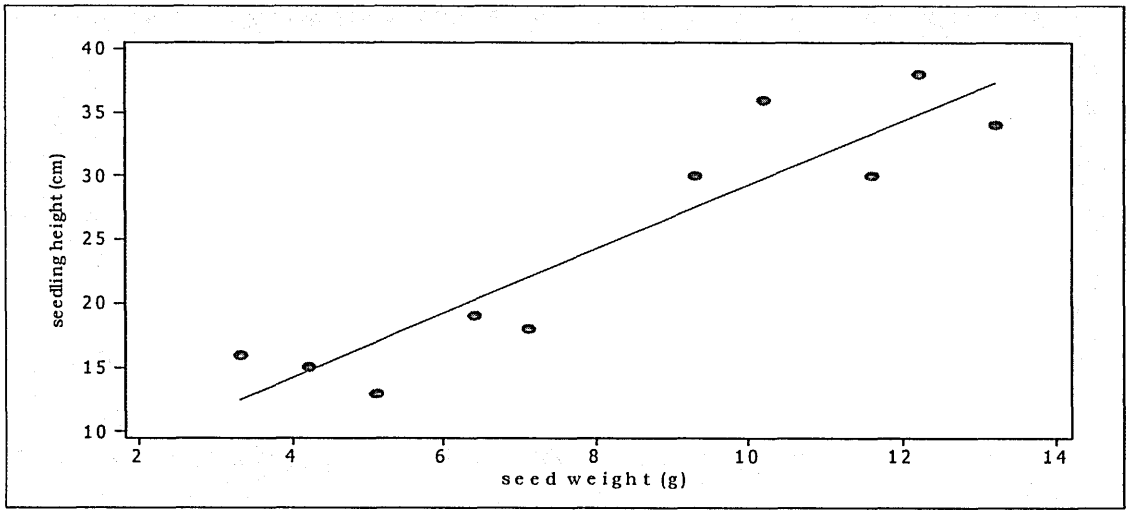

Figure A2.2 Scatter plot with fitted regression line for seedling height versus seed weight

The fitted line indicates that there might be a positive regression between seed weight and seedling height since the gradient is positive. This could be confirmed with a simple linear regression analysis produced by Minitab software (Table A2.2).

Regression analysis results for seedling height versus seed weight

The regression equation is:
seedling height = 4.11 + 2.52 seed weight

From this formula, seedling height could be predicted from the seed's weight. The regression table for the above experiment is shown in Table A2.2. It gives information on the coefficient, coefficient standard error, T and P values for the β_0 (constant) and β_i (the gradient of the predictor variable seed weight). These are defined as in Table A2.2.

Table A2.2 Regression analysis result for seed weight versus seedling height

Predictor	Coef	SE Coef	T	P
Constant	4.113	3.302	1.25	0.248
seed weight	2.5165	0.371	6.79	0.000

R-Sq = 85.2%

Analysis of Variance

Source	**DF**	**SS**	**MS**	**F**	**P**
Regression	1	708.06	708.06	46.11	0.000
Residual Error	8	122.84	15.36		
Total	9	830.90			

The relationship between the two variables (whether the relationship is positive or negative) could also be investigated by using the regression coefficient. If the regression coefficient is positive, then there is a positive relationship between the two variables. If the coefficient is equal to 0.5, for example, then that for one unit increase in the predictor variable, the response variable would increase by 0.5 units. If the coefficient is -0.5, then for one unit increase in the predictor variable, the response variable would decrease by 0.5 units. In this example, the direction of the relationship between seed weight and seedling height is determined more specifically by looking at the coefficient for seed weight. The positive value of the coefficient for seed weight (2.5165) shows that there is a positive linear relationship between seed weight and seedling height. Further the seedling height will increase 2.5165cm when the seed weight increase is one gram.

The regression output also shows if the model can predict one variable based on another variable by the significance level of the overall F of the model from the ANOVA table. If the significance is 0.05 (or less), then model is considered significant. In this example, the p-value is 0.000 which is less than 0.05. This indicates that seed weight has a significant positive linear regression relationship with seedling height at the 5% significance level. The T-value provides information on individual variable whilst, the F value for overall model. In this experiment, the p-values for these two are the same since there is only one variable.

The regression analysis also provides the coefficient of determination, R^2 (R-sq in Table A2.2), which is the proportion of the variation in the response variable explained by the regression model. It can range from zero to one and is often expressed as percentage. When the R^2 shows 100%, there is a perfect fit of the linear model. A R^2 value of above 60% is usually regarded as

acceptable for many biological applications (Zou *et al.*, 2003). In the above example, the R^2 value is 85.2%, indicating a well-fitting linear model.

A2.2.2 Assumptions of regression analysis

As in the ANOVA, there are three main assumptions in regression analysis. They are independence of the observations, normality and homoscedasticity.

A2.2.2.1 Independence of the observations

The first assumption is an independence of the observations. This form of violation resides in the lack of independence of observations. This lack of independence of the errors can bias the estimation of significance tests and cause the R^2, F and t values to become inflated.

A2.2.2.2 Normality

The second assumption is normality of the residuals. When there are violations of assumption of normality of the residual, the estimation of significance becomes impaired. If the residual distribution is normally distributed, the analyst can determine where the level of significance or rejection regions begin. This can be tested by applying an Anderson-Darling test on residual as described in Section A1.3.4.1.

A2.2.2.3 Homoscedasticity

Another basic assumption of regression analysis is equality of the error variance along the predicted line (homoscedasticity) (Haining, 1997). Heteroscedasticity is assessed by looking at the residual values plotted against the fitted values. For homoscedasticity, the distance between the line y = 0 and the residual will be equal. If not then data transformation need to be carried out. For the above example, the residual against fitted values plot shows that the residuals are homoscedastic (Figure A2.3).

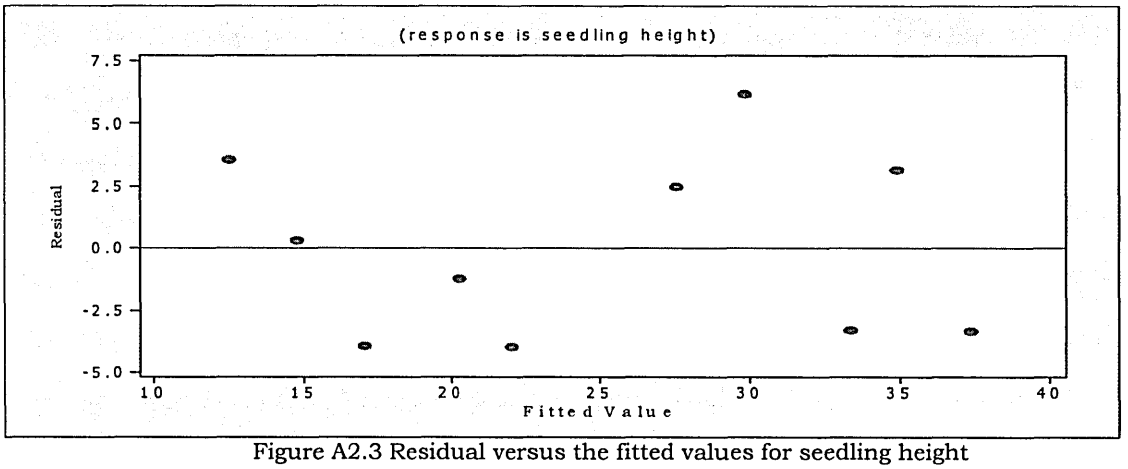

Figure A2.3 Residual versus the fitted values for seedling height

In this thesis, linear regression analyses were carried out to find the fitted values for unobserved treatment combinations in the fractional experiments. Where regression analyses were used, the diagnostic checks were performed.

A2.2.3 Multiple linear regression

Multiple linear regression is an extension of simple linear regression, by including several predictor variables. The multiple regression analysis equation is of the form

$$Y_i = \beta_o + \beta_1 X_{1i} + \beta_2 X_{2i} + \beta_3 X_{3i} + \ldots\ldots + \beta_k X_{ki} + \epsilon_i$$

where Y_i is the i^{th} value of response variable, y (i = 1, 2,..n) and n is number of observations

X_1, X_2, ..., X_k = predictor variables, X_{ij} = i^{th} observation of the j^{th} predictor variable (i=1,2...n; j=1,...k)

β_j = gradient for X_j

β_o = a constant as in the simple linear regression

ϵ_i = i^{th} independently and identically distributed error term, IID $N(0, \sigma^2)$

To continue with the previous example, assume that there is another predictor variable (seed moisture content) (Table A2.3). To predict a seedling's height from the moisture content and weight of the seed, standard multiple regression could be used in which weight and moisture content were the predictor variables and seedling height was the response variable. The resulting output indicates how the seedling height varies by knowing a seed's moisture content and weight. The regression analysis equation and result table (Table A2.4) are shown below.

Table A2.3 Data set for seed weight, moisture content and seedling height

Seed weight (g)	Seed moisture content (%)	Seedling height (cm)
3.3	78	16
7.1	62	18
13.2	46	34
10.2	89	36
9.3	64	30
6.4	74	19
4.2	81	15
5.1	92	13
12.2	64	38
11.6	68	30

Table A2.4 The regression analysis results

Predictor	Coef	SE Coef	T	P
Constant	-8.08	11.25	-0.72	0.496
seed weight	2.8377	0.4618	6.14	0.000
seed MC	0.1328	0.1174	1.13	0.295

R-Sq = 87.5%

Analysis of Variance

Source	DF	SS	MS	F	P
Regression	2	727.05	363.52	24.50	0.001
Residual Error	7	103.85	14.84		
Total	9	830.90			

In addition to the predictive value of the overall model, standard multiple regression indicates how well each predictor variable predicts the response variable, controlling for each of the other predictor variables. In the given example, the regression shows how moisture content predicted a seedling's height, controlling for seed weight, as well as how seed weight predicted a seedling's height, controlling for seed moisture content. The significance level associated with moisture content will inform if moisture content was a 'significant' predictor of germination. As previously, significance levels of 0.05 or lower are considered significant, and significance levels between 0.05 and 0.10 are considered marginal.

The significance levels given for each predictor variable indicates whether it is a significant predictor of the response variable, over and above the other predictor variables. Because of this, a predictor variable that is a significant predictor of a response variable in simple linear regression may not be significant in multiple regression (i.e., when other predictor variables are added into the formula). This could happen because the variance that the first predictor variable shares with the response variable could overlap with the variance that is shared between the second predictor variable and the response variable. Consequently, the first predictor variable is no longer uniquely predictive and thus would not show up as being significant in the multiple regression. Because of this, it is possible to get a highly significant overall model, but have none of the predictor variables significant.

For above experiment the regression model is:
seedling height = - 8.08 + 2.84 seed weight + 0.13 seed moisture content

The F ratio of 24.5 (p=0.001) shows that this model is significant however, the regression table shows that only seed weight is a significant predictor variable (p = 0.000) of seedling height but not seed moisture content (p = 0.295). This is a disadvantage of multiple regression analysis where it includes both significant and insignificant predictor variables in the model. This is especially the case when there are many predictor variables in the model. However this problem could be solved by using an algorithm to identify a model with only the significant effects efficiently.

A2.2.4 Efficient way to determine redundancy in regression model

Multiple regression analysis could be used to fit main effects and their interactions in a designed experiment. However, as mentioned above, redundancy in regression model is important when there are many predictor variables in the experiment. Stepwise multiple regression analysis provides the opportunity to check for model redundancy and to obtain a simple model which will contain only the significant effects (Draper and Smith, 1998). Three options are considered in the stepwise multiple regression analysis. To show this, an illustrative example with three factors at two levels each with two replicates with Y as response variable is considered (Table A2.5). The regression table is shown in Table A2.6.

Table A2.5 Illustrative experiment for multiple regression analysis

A	B	C	Y (Replicate 1)	Y (Replicate 2)
-1	-1	-1	-17.0	-11.0
1	-1	-1	14.0	21.0
-1	1	-1	-16.0	-17.0
1	1	-1	14.0	13.0
-1	-1	1	-10.0	-16.0
1	-1	1	-8.0	-4.0
-1	1	1	-5.0	-18.0
1	1	1	-10.0	-3.0

The 'full' model with all main effects and two-way interaction is:

Y = - 4.56 + 9.19 A - 0.69 B - 4.69 C - 0.44 AB - 6.19 AC + 0.94 BC

Table A2.6 The regression analysis output for the full model

Predictor	Coef	SE Coef	T	P
Constant	-4.563	1.114	-4.10	0.003
A	9.187	1.114	8.25	0.000
B	-0.687	1.114	-0.62	0.552
C	-4.688	1.114	-4.21	0.002
AB	-0.437	1.114	-0.39	0.704
AC	-6.188	1.114	-5.56	0.000
BC	0.937	1.114	0.84	0.422

R-Sq = 92.9%

Table A2.6, shows that only the main effects A and C and the two-way interaction of AC are significant. Hence the full model contains many insignificant variables. Three algorithms designed to identify efficiently a subset of the predictor variable that provide a reasonable multiple linear regression are considered below.

Forward selection Option: The first option for identifying multiple regression model redundancy is using forward selection. Forward selection starts out with no predictors in the model and adds one variable at each step. Each of the available predictors is evaluated with respect to how much R^2 increases by adding it to the model. The one which will most increase R^2 is added (with a p-value < 0.05). If no predictors meet this criterion for entry, the analysis will stop. If a predictor is added, then the second step progresses including this variable to those already in the model. If any satisfy the criterion for entry, the one which most increases R^2 is added. This procedure is repeated until there remain no more predictors that are eligible for entry. The forward selection analysis for above experiment is shown in Table A2.7. It shows a forward selection analysis with alpha-to-enter of 0.01, which specifies the significance level for entry into the model.

Table A2.7 The regression analysis result for option 'forward selection'

Step	1	2	3
Constant	-4.563	-4.563	-4.563
A	9.2	9.2	9.2
T-Value	4.02	5.63	8.93
P-Value	0.001	0.000	0.000
AC		-6.2	-6.2
T-Value		-3.79	-6.01
P-Value		0.002	0.000
C			-4.7
T-Value			-4.56
P-Value			0.001
R-Sq	53.64	77.97	91.93

The first step involves only main effect A since it has the largest R^2 (53.64) of any individual predictor variable. This is consistent with the results in Table A2.7 that shows that A has the smallest p-value. The second step involves adding the two-way interaction AC. The value of R^2 for the model with these two variable is 77.97. This step will be potentially repeated until all the main effects and interactions are included in the model.

In this experiment, the best model from this method would be the one with main effects A, C and two-way interaction AC which have p-value less than 0.05. Adding three further variables only increased the value of R^2 from 91.93 to 92.91.

Backward elimination option: A backward elimination analysis starts out with all of the predictors (factors and chosen interactions) in the model. At each step we evaluate the predictors which are in the model and eliminate one by one those that meet the criterion for removal (those with the p-value >0.05 and the largest p-value first). An example of using backward elimination for the above experiment is shown in Table A2.8. The alpha-to-remove value specifies the significant level for staying in the model.

In the first step, all predictors were included in the model. In the second step, the two-way interaction AB has the largest p-value (0.704) and therefore is removed. Removing AB would reduce least the value of R^2 (in this case 92.91% to 92.79%), so AB was removed. In the third step, the main effect of B was removed since it has the largest p-value (0.533). Removing B from the model changed the R^2 value from 92.79% to 92.49%. In step four, the two-way interaction BC was removed following the same criterion. This will leave a final model with main effects A, C and two-way interaction AC with p-value less than 0.05 with a R^2 value of 91.93%.

Table A2.8 Regression analysis result for option 'backward elimination'

Step	1	2	3	4
Constant	-4.563	-4.563	-4.563	-4.563
A	9.2	9.2	9.2	9.2
T-Value	8.25	8.62	8.86	8.93
P-Value	0.000	0.000	0.000	0.000
B	-0.7	-0.7		
T-Value	-0.62	-0.65		
P-Value	0.552	0.533		
C	-4.7	-4.7	-4.7	-4.7
T-Value	-4.21	-4.40	-4.52	-4.56
P-Value	0.002	0.001	0.001	0.001
AB	-0.4			
T-Value	-0.39			
P-Value	0.704			
AC	-6.2	-6.2	-6.2	-6.2
T-Value	-5.56	-5.81	-5.97	-6.01
P-Value	0.000	0.000	0.000	0.000
BC	0.9	0.9	0.9	
T-Value	0.84	0.88	0.90	
P-Value	0.422	0.400	0.385	
R-Sq	92.91	92.79	92.49	91.93

Fully stepwise selection option: Fully stepwise selection analysis starts out as with forward selection. In the second step, another effect that meet the entry criterion ($p<0.05$) will be included to the model if appropriate. This will then be followed by re-evaluating the model containing both

effects for removal and if any are eligible for removal, the one whose removal would least lower R^2 is removed. This step then will be followed by adding the next effect that meet the criterion for entry, and again this will be followed by re-evaluating the model. This will be repeated until all effects that are eligible for entering are included in the model. The example is shown in Table A2.9. Factor A was included as in the forward selection option and then followed by two-way interaction AC and factor C. Since only main effects A, C and two-way interaction AC were significant, these were the only effects in the final model.

Table A2.9 The regression analysis result for 'fully stepwise selection'

Step	1	2	3
Constant	-4.563	-4.563	-4.563
A	9.2	9.2	9.2
T-Value	4.02	5.63	8.93
P-Value	0.001	0.000	0.000
AC		-6.2	-6.2
T-Value		-3.79	-6.01
P-Value		0.002	0.000
C			-4.7
T-Value			-4.56
P-Value			0.001
R-Sq	53.64	77.97	91.93

Alpha-to-Enter: 0.15 Alpha-to-Remove: 0.15

All the above options of stepwise regression analyses provided the same model. However, it may not be the case always. Therefore, it is up to the experimenter to select the algorithm that suits the experiments. In this thesis, fully stepwise regression analysis was used since it comprises both forward selection and backward elimination methods in it. It was used for all the fractionally replicated experiments to find a model with all the significant effects. The regression equation is used to calculate all the fitted values for the full experiment. The best treatments for the fitted values were obtained by ranking the treatment combinations. The fitted values were also compared with the observed values from the full experiment using Spearman's rank correlation (Section A2.3.2).

A2.2.5 Binary logistic regression

Binary logistic regression analysis is used when the response variable follows a binomial distribution (Sokal and Rohlf, 1995). This analysis is suitable when researchers want to analyse whether some event occurred (success) or not (failure), such as viability of a plant material after cryopreservation. Values are coded "yes" (coded 1) for survived material and "no" (coded 0) for non-survived materials.

There are two major problems with using a linear regression model with binary data:

1. The observed values are not normal distribution since they take only two values
2. The predicted probabilities can be greater than 1 or less than 0, which can be a problem if the predicted values are used in a subsequent analysis.

The logit distribution defined as $\log_e \frac{P}{1-P}$, where p=probability (success), constrains the estimated probabilities to lie between zero and one. The binary logistic regression model has the form;

$$\log_e \frac{P}{1-P} = \beta_o + \beta_1 X_{1i} + \beta_2 X_{2i} + \beta_3 X_{3i} + \ldots\ldots + \beta_k X_{ki} + \epsilon_i$$

which is similar to the multiple linear regression model. In common with the multiple linear regression model, redundancy can exist and how a parsimoniam model can be found is explained next.

The logistic regression analysis calculates a null deviance which summarises the fit of a logistic model that just includes an intercept (Rice, 1994). This model would predict a constant value for the response variable regardless of the value of the predictor variable. By looking at the change in the deviance (deviance difference) when a predictor variable is added to the model, it can be determined whether or not that predictor variable is adding significantly to the predictive ability of the model. For example, the difference between the null deviance and the deviance explained represents the effect of adding a single predictor variable to the logistic model. The increase in deviance explained in logistic regression can be compared to a Chi-square (X^2) distribution to determine statistical significant. The degrees of freedom for the X^2 is equal to the number of predictor variable added to the model.

Logistic regression was used in preference to the often-used arcsine transformation or ANOVA as these assume normality and require a large number of replicates. For example, consider an experiment with two factors (A and B each with three levels) and number of viable seeds from a total of 10 as the response variable. The experimental design and observed values are shown in Table A2.10. The binary logistic regression analysis result for above experiment is shown in Table A2.11.

Table A2.10 Design of experiment for factors A and B

Factor A	**Factor B**	**No of viable seed**	**Total seed**
1	1	5	10
1	2	4	10
1	3	7	10
2	1	5	10
2	2	6	10
2	3	3	10
3	1	4	10
3	2	5	10
3	3	7	10

Table A2.11 Analysis of deviance for factors A and B

Model	Deviance explained	DF	Deviance difference, G*	DF * difference	P-value
1. Null	0	0			
2. A	0.820	1	0.820	1	>0.05
3. $A + A^2$	5.111	2	4.291	1	<0.05
4. $A + A^2 + B$	6.960	3	1.849	1	>0.05
4. $A + A^2 + B + B^2$	17.982	4	11.022	1	<0.001
5. $A + A^2 + B + B^2 + AB$	26.617	5	8.635	1	<0.01

DF= degrees of freedom, A^2= quadratic function of factor A, B^2 = quadratic function of factor B.

The null hypothesis is that there is no effect of predictor variable on this response variable. The deviance difference, G* is calculated as the difference in deviance explained when a variable is added to the model. For example, in Table A2.10, the value of G* for the model containing A is the difference between G value of Null model (with no effects) and model two (with A) which is 0.820 - 0 = 0.820. The degrees of freedom difference is the difference between degrees of freedom of the previous and present models. Therefore, for model two, the degrees of freedom difference between null model and model one is 1-0 = 1. The critical value for the Chi-square distribution with one degree of freedom to be significant at the 5% level is 3.841, at 1% level is 6.635 and at 0.1% level is 10.827 (Murdoch and Barnes, 1986).

The above analysis showed that adding factor A to the Null model is not significant. However adding its quadratic function to the linear term is significant (G* = 4.291, DF*=1, $p<0.05$). When a quadratic function of an effect is included in a model, its linear term also needs to be included though it has no significant effect.

Adding factor B to model three does not significantly affect the model predicting the number of viable seeds. However, the quadratic function of B has a significant effect on the model (G*11.022, DF*=1, $p<0.001$). Fitting the full model involving A, B, their quadratics and their interactions also had a significant effect on the model predicting number of viable seed (G* =8.635, DF*=1, $p<0.01$) compared with the model without the interaction. Therefore, the final model for this analysis contains factors A, B, their interaction and quadratic effects. This model is used to find the fitted values similar to the stepwise regression analysis.

In this thesis, logistic regression analysis was used to analyse data in Section 7.4 and Chapter eight, since the response variable for these experiments (viable embryos or shoot-tips after cryopreservation) followed a binomial distribution and therefore could not be analysed using ANOVA or linear regression analysis.

5.3 Correlation Analysis

A2.3.1 Product-moment linear correlation (Pearson r)

Product-moment or Pearson's correlation coefficient (r) measures the degree to which two quantitative variables are linearly associated. The correlation coefficient ranges between -1 (perfect negative linear association) and +1 (perfect positive linear association) inclusive. A coefficient value of zero indicates that there is no linear association between the two variables. The formula for r is;

$$r = \frac{\sum_{i=1}^{n}\left(x_i - \bar{x}\right)\left(y_i - \bar{y}\right)}{\sqrt{\sum_{i=1}^{n}\left(x_i - \bar{x}\right)^2 \sum_{i=1}^{n}\left(y_i - \bar{y}\right)^2}}$$

where x_i = i^{th} observation of predictor variable, x

y_i = i^{th} observation of response variable, y

$\bar{x}$ = mean of x variable

$\bar{y}$ = mean of y variable

The significance of a correlation coefficient of a particular magnitude will change depending on the sample size. The test of significance is based on the assumption that the distribution of the residual values (i.e., the deviations from the regression line) for the dependent variable y follows a normal distribution, and that the variability of the residual values is the same for all values of the independent variable x. These can be diagnosed using methods described in Section A2.2.2.

Example of correlation analysis

Consider again the data in Table A2.1 shown below again in Table A2.12 for covariance. A correlation analysis could be used to investigate the strength of the linear relationship between these two variables.

Table A2.12 Data set for correlation analysis example experiment

x	y
Seed weight (g)	**Seedling height (cm)**
3.3	16
7.1	18
13.2	34
10.2	36
9.3	30
6.4	19
4.2	15
5.1	13
12.2	38
11.6	30

The correlation coefficient calculated using above formula showed that

$$r = \frac{281.36}{\sqrt{111.804 * 830.9}} = \frac{281.36}{\sqrt{92897.9}} = \frac{281.36}{304.792} = 0.923$$

This indicates a positive correlation between seed weight and seedling height with a correlation coefficient r of 0.923. The Pearson correlation analysis output by Minitab also revealed the similar result with r = 0.923, (p=0.000). Therefore, it can be concluded that seeds with greater weight will produce seedlings with greater height. Note that for a simple linear regression the value of R^2 is the square of Pearson's correlation coefficient. For this example, R^2 is 85.2% = 0.852, which is equal to square of 0.923 (Section A2.2.1, Table A2.2).

A2.3.2 Spearman's rank correlation

In some applications, the assumption for Pearson's correlation coefficient are violated. In these cases, the ranks of the experimental treatment combinations can be compared using Spearman's rank correlation to investigate if they are in agreement or not (Quinn and Keough, 2002). Spearman's rank correlation is a distribution-free analogue of Pearson's correlation analysis (Sokal

and Rohlf, 1995) meaning that it does not assume normality of data. It can be applied to compare two independent variables, each at several levels. Unlike regression, Spearman's rank correlation works on ranked (relative) data, rather than directly on the data itself. Basically, R_s is the Pearson's correlation where the values are converted to ranks before computing the coefficient.

Spearman's method works by assigning a rank to each observation in each group separately and calculating the sums of the squares of the differences in paired ranks. The coefficient is used to discover the strength of a link between two sets of data. Each variable is ranked separately by putting the values of the variable in order and numbering them; the lowest value is given rank one, the next lowest is given rank two and so on. If two data values for the variables are the same, their ranks are averaged, so if they would have been ranked 10 and 11 then they both receive rank 10.5.

Spearman's rank correlation coefficient R_s, like all other correlation coefficients, takes a value between −1 and +1 inclusive. A positive correlation is one in which the ranks of both variables increase together. A negative correlation is one in which the ranks of one variable increase as the ranks of the other variable decrease.

Example of Spearman's rank correlation: Consider the data set in Table A2.13. The steps to calculate the R_s value is show below:

Table A2.13 Data Table for Spearman's Rank Correlation

Seedling height (cm)	**Rank**	**Seed weight (g)**	**Rank**	**Modulus of Difference between the ranks |d|**	**D^2**
16	3	3.3	1	2	4
18	4	7.1	5	1	1
34	8	13.2	10	2	4
36	9	10.2	7	2	4
30	6.5	9.3	6	0.5	0.25
19	5	6.4	4	1	1
15	2	4.2	2	0	0
13	1	5.1	3	2	4
38	10	12.2	9	1	1
30	6.5	11.6	8	1.5	2.25
					$\sum d^2 =$ 21.5

- Data sets are ranked by giving the ranking 'one' to the smallest number in a column, 'two' to the second smallest value and so on.
- The difference in the ranks (d) which the difference between the ranks of the two values on each row of the table are calculated. For example in column one of Table A2.13, the difference between rank three (seedling height) and rank one (seed weight) is two.
- The differences are square (d^2) to remove negative values and then summed. All the d^2 values were calculated by adding up all the values in the column. In the above example this is 21.5. Multiplying this by six gives 129.
- The value n is the number of paired observation. This, in the above example is 10. Substituting these values into $n^3 - n$ we get 1000-10 = 990.

- The Spearman's rank correlation coefficient (R_s) was calculated using the formula below:

$$R_s = 1 - \frac{6\sum d^2}{n^3 - n}$$

- R_s = 1 - (129/990) which gives a value for R_s= 1 – 0.130 = 0.870.

The closer R_s is to +1 or -1, the stronger the correlation. A perfect positive correlation is +1 and a perfect negative correlation is -1. The R_s value of 0.870 suggests a very strong positive relationship. Minitab also gave similar output for Spearman's rank correlation coefficient which was 0.869. The critical value for this to be significant is 0.746 at 1% significance level. Since for this example the value is 0.870, it indicates that the positive rank correlation between seedling height and seed weight was significant.

Spearman's rank correlation analysis was used in this thesis to compare the ranks of treatment combinations for all the response variables obtained using full factorial and fractional replicates and Taguchi full and fractional experiments.

A2.4 Analysis of Covariance (ANCOVA)

Analysis of Covariance (ANCOVA) is a technique that sits between analysis of variance and regression analysis (Rutherford, 2000). Analysis of covariance is used when the response variable y, in addition to being affected by the controllable treatments, is also linearly related to another continuous variable x. The analysis of covariance is useful in several types of research situations. It has a number of purposes but the two that are, perhaps, of most importance for this thesis are:

1. to increase the precision of comparisons between groups by accounting for variation on important predictive variables;

2. to 'adjust' comparisons between groups for imbalances in important predictive variables between these groups. This is done by building a scatter diagram from simple linear regression.

Point two is specific for ANCOVA which is the 'adjustments' for other variables is often encountered in the biological experiments (Rutherford, 2000). The method of ANCOVA allows the analyst to make comparisons between groups that are comparable with respect to some important variable, often referred to as a covariate (Rutherford, 2000).

Example of ANCOVA

Consider an experiment where the weight was found for 10 and eight randomly chosen seeds of two different species (Table A2.14). The height of the related seedling is used as the response variable. ANCOVA is used to determine if this relates to seed weight and if the two species have different seedling height for the same seed weight.

Table A2.14 Data set for correlation analysis example experiment

Species A Seed weight (g)	Species A Seedling Height (cm)	Species B Seed weight (g)	Species B Seedling Height (cm)
3.3	16	12.0	44
7.1	18	4.2	25
13.2	34	9.7	38
10.2	36	14.8	51
9.3	30	11.8	44
6.4	19	9.4	38
4.2	15	8.5	35
5.1	13	9.8	39
12.2	38		
11.6	30		

The ANCOVA table is shown in Table A2.15.

Table A2.15 Analysis of Variance Table for covariance analysis

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Seed weight	1	1622.5	1114.7	1114.7	135.15	0.000
Species = B	1	407.4	407.4	407.4	49.40	0.000
Error	15	123.7	123.7	8.2		
Total	17	2153.6				

R-Sq = 94.26%

The Regression equation is

Height = 4.26 + 2.50 seed weight + 9.94 (species = B)

Interpretation of ANCOVA

ANCOVA showed that covariate seed weight [F(1,15)=135.15, p=0.000] have a significant effect on the seedling height. Species [F(1,15)=49.4, p=0.000] also has a significant effect on the seedling height. Therefore species B has a different effect on the seedling height compared to species A for a fixed seed weight.

Table A2.16 Regression analysis table for covariance analysis

Predictor	Coef	SE Coef	T	P
Constant	4.256	1.995	2.13	0.050
Species = B	2.49992	0.2150	11.63	0.000
Seed weight	9.939	1.414	7.03	0.000

The regression analysis showed that species B increased seedling height by 51.5cm (coefficient value) compared to Species A for the same seed weight. This is illustrated in the scattered plot with fitted regression lines for species A and B (Figure A2.4).

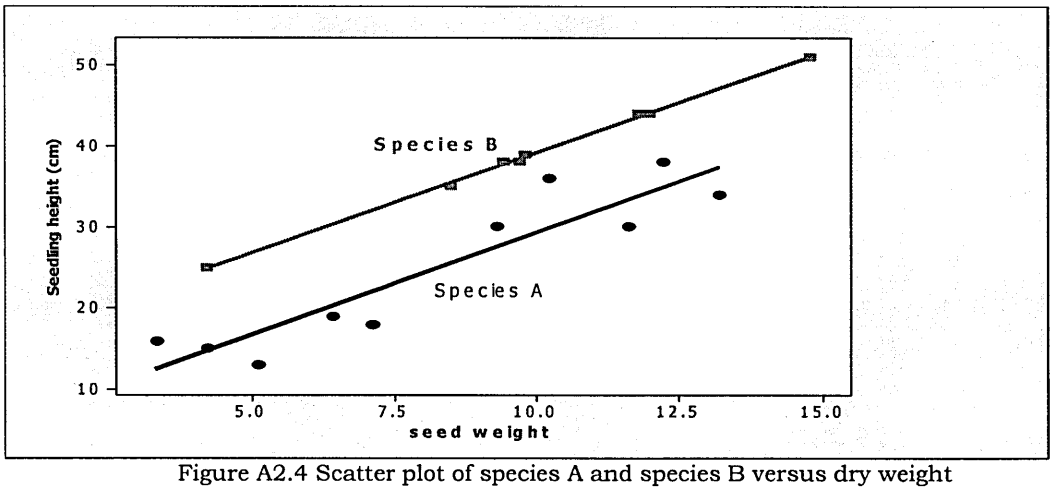

Figure A2.4 Scatter plot of species A and species B versus dry weight

ANCOVA was used in Section 6.5 of this thesis, to evaluate the response variables on three different types of germination substrates where desiccation time and warming rate were selected as covariates.

A2.5 Summary

This chapter concludes the reviews of the statistical and experimental design methods and applications. The thesis will now progress with the application of novel experimental designs and analysis to rainforest tree germplasm cryopreservation protocol development.

For the experiments in Chapters six and seven in this thesis, full factorial and Taguchi full experiments were conducted to compare 'mean' and 'SNR' analyses. The experiments were analysed with ANOVA to investigate main effects and two-way interactions. Treatment combinations using mean and SNR were ranked and compared using Spearman's rank correlation analysis (Quinn and Keough, 2002).

The suitable subsets of fractional and Taguchi designs were selected for each experiment conducted. Stepwise regression analysis (Draper and Smith, 1998) was used to find suitable models from these fractionally replicated experiments. The stepwise regression model was used to estimate the mean response and SNR for all treatments. The fitted values were ranked and Spearman's rank correlation analysis was used to compare these with those of the observed mean and SNR values respectively from the full experiment to justify the model. Fitted mean and SNR values were also compared using Spearman's rank correlation.