Physiology Project

Cryopreservation of *Podocarpus elongatus* (Ait.) L'Hérit. ex Pers. and *Podocarpus falcatus* (Thunb.) R. Br. ex Mirb. zygotic embryos

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Botany Honours 1994
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ABSTRACT

The aim of this project was to develop a protocol for the cryopreservation of the zygotic embryos from the putatively recalcitrant seeded *Podocarpus elongatus* (Ait.) L'Hérit. ex Pers. *Podocarpus falcatus* (Thunb.) R. Br. ex Mirb. was going to be used as a comparative, orthodox seeded species but its germination and development was too slow for the time that was available. The most significant discovery was that embryos from both species were very sensitive to hypochlorite sterilisation. Callous growth, after no sterilisation or mild sterilisation (0.175% hypochlorite), was achieved on Murashige and Skoog (1962) medium supplemented with sucrose, Naphthalene Acetic Acid (NAA), 6-Benzylaminopurine (BAP) and Giberellic acid 3 (GA_3). *P. elongatus* could not survive flash drying to a moisture content of below 30% (WMB) and was intolerant to freezing in liquid nitrogen (-196°C). There are indications, however, that the embryos become quiescent after treatment and resume growth after an extended period of time. This was concluded from the observation that an embryo that had been dried to a moisture content of 35% (WMB) followed by freezing in liquid nitrogen developed callous tissue after a period of 34 days. The effect of two cryoprotectants on the survival of flash dried and frozen embryos were studied. The results from these experiments were inconclusive.

INTRODUCTION

The aim of this project was to develop a protocol for the cryopreservation of *Podocarpus elongatus* and *Podocarpus falcatus* zygotic embryos. Yellowwoods where chosen for this study for a number of reasons. Firstly, a study such as this uses a large amount of seed material and so we were unable to obtain sufficient quantities of seed of any of the locally available recalcitrant bulbous plants such as is found in the Amarilidaceae. Furthermore it was suggested that *P. elongatus* produces recalcitrant seeds (Farrant, pers. comm.). *P. falcatus* produces orthodox seed (Farrant, 1982) and so this species was chosen as a comparative species whose results could be compared with those obtained from the study of the *P. elongatus*.

Podocarpus elongatus (Ait.) L'Hérit. ex Pers. is commonly called the Breede River yellowwood. It is the smallest of the South African yellowwoods (Coates-Palgrave, 1983) growing to between 3 and 6 meters tall. This species is restricted to the winter rainfall region of the western Cape (Figure 1). It is also susceptible to fire damage and so one can see that this tree could easily become threatened considering the fact that accidental fires are occurring more and more frequently.

Podocarpus falcatus (Thunb.) R. Br. ex Mirb., the Outeniqua yellowwood, is a medium to large tree that can grow to a height of 20 to 60 meters. It naturally grows in the southern Cape (Figure 1) and is often used horticulturally as a roadside tree in towns. Further, it is a good timber tree yielding high quality, straight grained timber (Coates Palgrave, 1983).

P. elongatus and *P. falcatus* have never been reported to have been subject to tissue culture before (George *et al.*, 1987) and so the initial step in the project was to find suitable media and growth conditions for these plants. Once this problem was solved, cryoprotection experiments could be performed.

THE THEORY OF RECALCITRANCE

Berjak *et al.* (1989) have reviewed the principles behind recalcitrant seed behaviour. The most important feature of recalcitrant seeds is that they do not undergo maturation drying during their final phase of development. This makes them intolerant of desiccation and they are often sensitive to chilling, making the long-term preservation of these seeds impossible when one is using traditional seed storage methods. It has been proposed that these seeds

Podocarpus falcatus *Podocarpus elongatus*

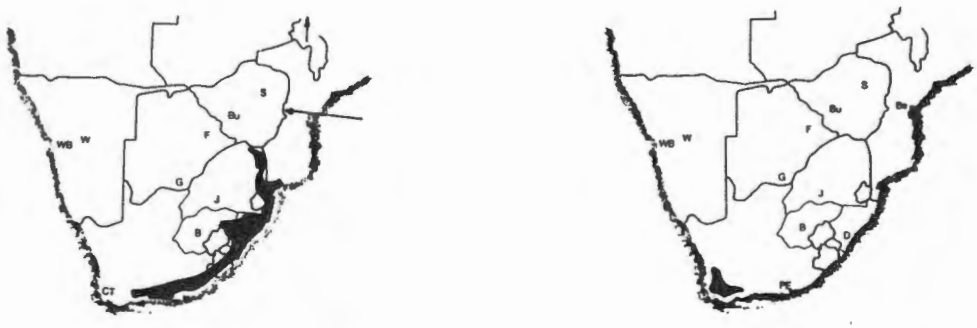

Figure 1: Natural distributions of the two species used in this study. (from Coates-Palgrave, 1983)

should be called homoiohydrous seeds which are contrasted with poikilohydrous (orthodox) seeds (Berjak *et al.*, 1989). It is pointed out that there are degrees of recalcitrance meaning that some species can tolerate moderate drying but not as much as is exhibited in orthodox seeds.

Plants that produce recalcitrant seeds occur in areas were seed dormancy or quiescence is not needed and so germination can occur as soon as the seed has matured. Germination can even occur while the seed is still attached to the parent plant (Berjak *et al.*, 1989). A further important consideration is that these seeds do not have a defined terminal point in their development, as is found in maturation drying of orthodox seeds. This means that different collections of seeds contain representatives that are in different physiological states and so it is difficult to get consistent results when one is studying these seeds.

Generally, the discussion concerning the desiccation sensitivity of the seeds is for intact seeds. Berjak *et al.* (1990) showed that intact seeds from *Landolphia kirkii* that were dried to a moisture content of 49% in a period of 20 days lost most of their viability while embryonic axes that had been excised from the seeds could be dried to a moisture content of 16% in the space of an hour and their viability remained undiminished. Electron microscopy revealed that general cellular integrity was retained after the flash drying procedure. This tolerance to flash drying is beneficial when one wants to cryopreserve the material because the lower moisture content makes freezing and thawing easier. This is explained in the next section.

THE THEORY OF CRYOPRESERVATION

When plant cells are frozen their survival predominantly depends on the behaviour of the water that is present within and surrounding the cells.

When water is cooled down it often remains liquid well below 0°C. This is because the liquid water needs stable nucleation sites in order to crystallise. Once crystallisation is initiated, the latent heat of fusion is released and the temperature of the sample will rise which prevents any further nucleation from occurring. This slow freezing process results in a solid filled with a few, large crystals. If the cooling rate is sufficiently rapid to actively remove the heat of fusion then new nucleation sites can develop and the sample will be filled with numerous, small ice crystals. A third possibility is if the water is cooled so rapidly that nucleation sites do not have time to develop the water becomes vitrified (glasslike), containing no ice crystals. Ice crystals grow at the expense of the water in their immediate surroundings and so the freezing front in a solution is preceded by a concentration gradient (Echlin, 1992).

Unfortunately the predictive models concerned with ice formation and its effects cannot be applied to biological systems because of the exceedingly complex aqueous environment found within cells (Echlin, 1992). Two processes cause significant damage to cells. The first is the solution effects observed just ahead of the freezing front which are responsible for dehydrating the cells, causing major changes in the biochemical state of the cell. The second process is the development in intracellular ice that can cause major disruption of delicate intracellular structures (Echlin, 1992). The disruption occurs when the cells are warmed up because in this state the small ice crystals become unstable and rearrange themselves into larger, more stable structures that can cause severe damage.

There is a trade-off between trying to freeze the cells fast enough to minimise cell damage through dehydration effects but still slow enough to prevent intracellular ice nucleation. This results in a survivorship curve in the shape of an inverted U with the optimal survival occurring at a cooling rate that is somewhere between very slow and very fast (Figure 2). The warming rate is also very important as can be seen in Figure 3. Rapidity of warming is necessary because this prevents excessive rearrangement from occurring which would result in the development potentially damaging ice crystals.

Cryopreservation involves the controlled freezing of the cells or tissue of interest and subsequent storage of this material for an extended period of time. It is vitally important

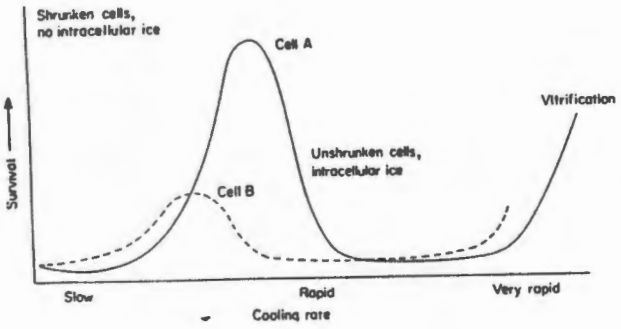

Figure 2: Diagrammatic representation of the effects of rate of cooling on two hypothetical cell types. (from Farrant *et al.*, 1977)

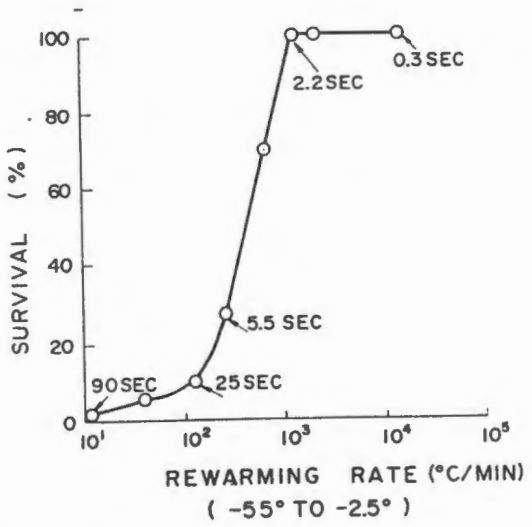

Figure 3: Effect of rewarming rate on the survival of cells immersed in liquid nitrogen. Times noted alongside the curve indicate the time required for the temperature to rise from -55 to -2.5°C. (from Sakai, 1985)

that the cells or tissues survive the freezing and thawing steps as well as not deteriorating while they are in the frozen state. Stanwood (1985) highlights some of the advantages of using liquid nitrogen (-197°C) for the storage of plant material. Firstly, all biochemical processes are significantly reduced and biological deterioration is virtually stopped. Further, storage in liquid nitrogen is a passive, reliable method of preservation because it relies purely on the occasional replenishment of the liquid nitrogen reservoir, there are no mechanical devices or reliance on electricity. The only requirement is a bulk supply of liquid nitrogen.

Cryopreservation techniques are most important for plants that produce recalcitrant seeds (Bajaj, 1985). This is because orthodox seed pose no storage problems when one uses traditional seed storage methods. By their very nature recalcitrant seed embryos are difficult to cryopreserve. This because of their large size which makes it difficult to cool and warm the embryos at required rapid rate (Bajaj, 1985) as well as the fact that they are intolerant to desiccation so they cannot be dried which would enhance the rates of cooling and warming. This led to the development various cryoprotection protocols but all of them are really empirical mixtures of chemicals purported to protect the tissues against freezing injury. There is very little knowledge about the actual physiological effect of these chemicals (Farrant pers. comm.).

Bajaj (1985) lists six factors that influence the survival of the tissue. These are: The genotype, size and stage of the development of the embryo, water content, presence of cryoprotectants, method and rate of freezing and, finally, the thawing rate and temperature. By taking the size of the embryo into account one should be able to develop generic protocols for the cryopreservation of plant embryos. Unfortunately, this ideal is confounded by the unique genetic composition of the plants which makes each situation a novel problem.

THE ROLE OF CRYOPRESERVATION IN PLANT CONSERVATION

Hamilton (1994) reviews of the present status of seed banks in conservation programs. It should be remembered that the cryopreservation of zygotic embryos is akin to long term storage of intact seed in seed banks and so the arguments presented in Hamilton's paper are applicable to both situations. Previously, ex situ collections of plants were used as a last resort method of preserving individual plant species. The emphasis has now shifted towards an integrated conservation approach that utilises ex situ collections as a necessary element of any conservation program. Models have been developed to predict the amount of genetic diversity present in any given plant population or ex situ collection. Hamilton (1994) points out that the presently used models are not actually suitable for ascertaining the genetic diversity and, more importantly, evolutionary potential of ex situ populations. The author contends that most of the available resources should be spent on primary conservation efforts in the form of development of natural reserve systems.

He does concede that ex situ collections do serve some important functions. In very restricted populations stochastic demographic events can severely threaten the survival of the population. The presence of a seed bank, although genetically restricted, can help to buffer the effect of these random events. Cryopreserved germplasm can also be used effectively in captive breeding programs by periodically infusing genetic material back into the living population from the cryopreserved population (Ballou, 1992). This can create an very large effective population that only has a few living representatives that require constant care and growing space. Vast amounts of genetic diversity can be stably stored in a small area with relative ease (Pence, 1990). Seed banks also have a potential role in education and research similar to that of botanic gardens for making plant material accessible to researchers and allowing cultivation for educational purposes. Given its severe limitations, ex situ conservation methods may be the only alternative in cases where other options have been

truly exhausted or it is certain that extant individuals will be extirpated (Hamilton, 1994). My one major criticism of these approaches is that these ex situ methods are actually true preservation, they prevent normal biological processes such as extinction from occurring.

Germplasm banks also serve a very valuable function in agriculture and horticulture. These banks can preserve novel lineages that have potential uses in various breeding programs. Parent genetic stock (wild-type) plants can also be preserved for future infusion into the inbred strains that are widely used in agriculture. Cryopreservation is often the only viable alternative for the preservation of many large seeded, recalcitrant tropical trees such as rubber and cacao (Grout *et al.*, 1983).

From the above discussion it can be seen that a seed bank is a dynamic entity that needs to be actively managed. It is not a passive collection of material that lies in store in the hope that it will be used in the future. Hamilton (1994) discusses some of the methods associated with efficient seed bank management. Small populations can have their fecundity severely reduced by large sample collecting for a seed bank. It is often better to make multiple small collections over an extended period of time as this will not jeopodise the survival of the population that one is trying to save. Careful note of the lineages of the different seeds needs to be made so that one can ensure that a maximum amount of diversity is maintained by sampling all of the lineages that are available, not just the most common one that would occur if all of the seeds were in one bulk store. Hamilton (1994) draws the analogy between an ex situ collection and an insurance policy. To be effectively covered continual payment of premiums must occur. That is to say the ex situ population needs to have a dynamic component to it, if it is going to effectively preserve the species in question.

The trees that used in the present study are not greatly threatened, although they are both protected species. The study was undertaken in order to gain deeper insight into methods of cryopreservation of recalcitrant seeds in general and may as such be applicable to other species of plants. This study is also acting as a pilot development of cryopreservation facilities in the laboratory. Hopefully it will set up guidelines for future research.

In this project 19 distinct experiments were performed. The results of earlier experiments governed my course of action in the later experiments and so the final results may look somewhat depauperate but they have been developed in the light of earlier failures. The format of this project report will be somewhat unorthodox. The methods section will

contain generally used methods as well as the methods used in the most successful experiments. This section will be followed by a section outlining the experimental procedure along with the presentation of preliminary results that governed the subsequent course of action. After this, the final results will be presented followed by a discussion and suggestions on how this study could be improved or developed in the future.

PLANT MATERIAL AND METHODS

PLANT MATERIAL

Podocarpus falcatus seed was collected from trees growing at the University of Cape Town (34°57'11"S, 18°27'50"E) near the sports centre on Woolsack Drive. The first collection was made on 20 June 1994 and a second collection was made on 16 August 1994. Some seeds where also collected from Constantia (34°01'S, 18°26'E) on 18 August 1994. The first batch of fruit where yellow with the skin just beginning to crack while the second batch of fruit where brown and wrinkled or half rotten, with a yellow-green colour. The seeds were depulped and then vigorously washed twice to remove any pulp that had not been removed in the initial step. They where then rinsed a number of times and allowed to dry on sheets of newspaper. Once the second batch of seeds where dry they where added to the first batch and mixed up so that a homogenous sample of the two batches was obtained. This was done so that the results could be considered constant over the rest of the experiments performed after the mixing. The seed was dusted with the fungicide Dithane M-45, placed in a paper packet and stored in a 10°C cold room.

Podocarpus elongatus seed was collected by Kirstenbosch Botanic Gardens staff on 2 June 1994 from the adult tree situated in the gardens (Accession number: 160/84). The fleshy aryl was removed and the seed was stored in a 10°C cold room. One thousand of these seeds were donated to this study by Kirstenbosch. Once at U.C.T., the seed was dusted with Dithane M-45, placed in a paper packet, and stored in a 10°C cold room.

Due to the restricted number of seeds available, all of the treatments used very small sample sizes. This means that the data obtained cannot be tested statistically although in many cases this is an empirical study whose results are plainly obvious and so one does not need to use statistics to get an idea of the significance of the results.

EXCISION OF THE EMBRYOS

The seeds were washed in water to remove the Dithane M-45 prior to excision of the embryo. During the excision process the blades and forceps were sterilised in 100% ethanol. Only intact embryos were used in the experiments and they were placed on moistened, sterile filter paper in sterile petri dishes until they were needed. There are indications that this treatment has no influence on the viability of the embryos. Chaudhury *et al.* (1991) obtained survival and germination of *Camellia sinensis* embryos stored on moist

tissue paper for 20 to 25 days. All of the manipulations occurred in an open laboratory except when the embryos were placed on the culture medium when a laminar flow bench was used.

The embryos of the seeds were removed by cutting into the seed with a scalpel and then gently prising the embryo away from the surrounding tissue. The hard shell of the *P. falcatus* seeds was cracked using a small vice that allowed for the even application of pressure ensuring that the soft flesh inside the seed was not damaged when the shell finally cracked.

CULTURE ENVIRONMENT AND MEDIUM

The general culture medium used, in this case called *Medium C*, consisted of full strength Murashige and Skoog (1962) medium (MS) with the addition of 3% sucrose, 0.7% agar, 1 mg/l 6-Benzylaminopurine (BAP), 1 mg/l Naphthalene Acetic Acid (NAA) and 1 mg/l Giberellic acid 3 (GA_3). The culture medium was adjusted to a pH of between 5.6 and 5.8 prior to sterilisation by autoclaving.

Cultured embryos were germinated in a 25°C constant temperature room with a 16/8 hour light/dark photoperiod.

MOISTURE CONTENT DETERMINATION

The moisture content of each embryo was ascertained gravimetrically by drying at 103°C for at least 18 hours. The moisture content was expressed on a *wet mass basis* and a *dry mass basis*. The latter is the usual method of reporting in the scientific literature. Figure 4 shows the relationship that allows one to convert wet mass basis to dry mass basis and *vice versa*.

The moisture content of the untreated, freshly excised embryos from the two species was determined using the method described above. 20 *P. elongatus* embryos were used in the determination while 15 were used for *P. falcatus*.

FLASH DRYING

The flash drying apparatus is shown in Figure 5. The apparatus utilised the departmental compressed air supply. Due to pressure fluctuations, a crude flow meter was used to enable a more or less constant airflow over time for both during the experiment as well as between

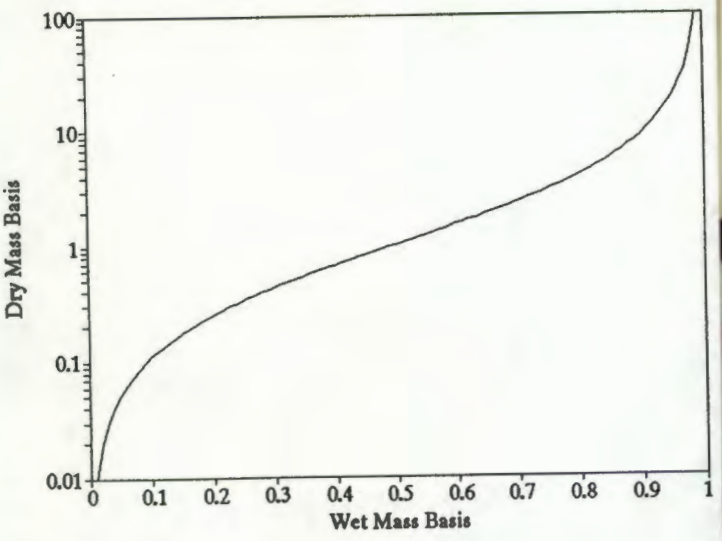

Figure 4: The relationship between the Dry Mass Basis and the Wet Mass Basis.

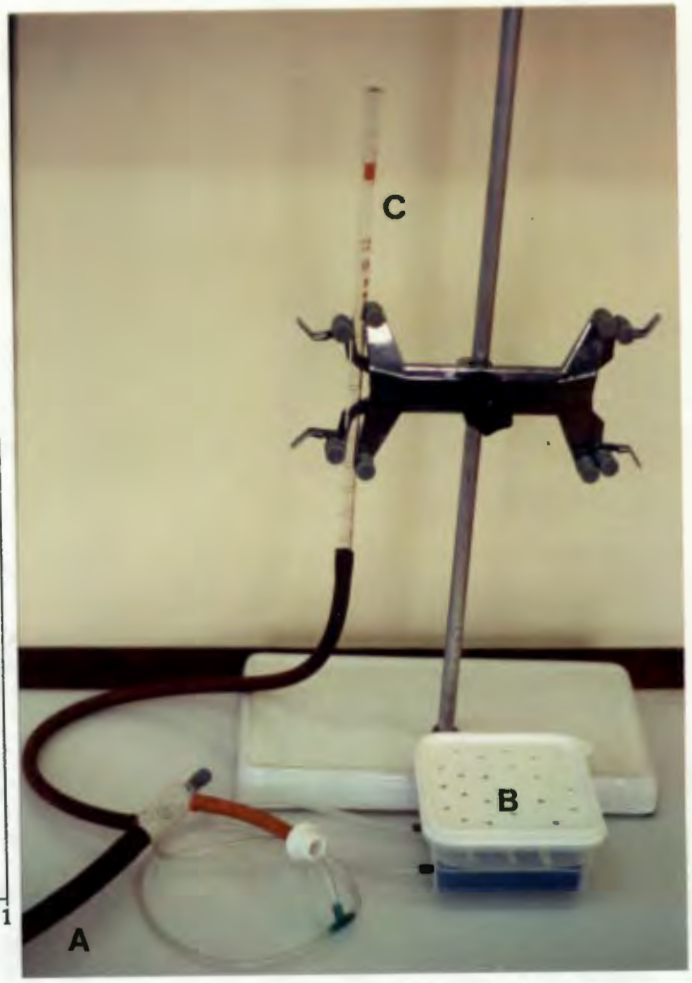

Figure 5: The flash drying apparatus used in this study. The components are: inlet for the compressed air (A), drying chamber (B) and flow meter (C).

each experiment. All of the embryos in each experiment where dried at the same time in the same drying box so that the embryos were progressively dried over time. Once the embryos were removed from the drying apparatus they were placed on damp filter paper so that the embryos had time to gradually rehydrate before the were placed on the culture medium. Chaudhury and Chandel (Date unknown) showed that desiccated neem seeds regained retained viability only if they were allowed to slowly rehydrate for 3 hours in 100% relative humidity at room temperature. In each experiment all of the embryos were placed on a moist piece of filter paper within one petri dish. This ensured that the rehydration environment was similar for all of the treated embryos.

FREEZING METHODS

There is considerable literature on the methods of freezing tissue for the purposes of

cryopreservation. In this study the following method of freezing of freezing was used: Six embryos from each drying treatment were placed in a small folded aluminium foil packet. The sides of the packet was gently squeezed together so that there was good contact between the walls of the packet and the embryos. This contact with the highly conductive aluminium would help to maximise the cooling rate. The top of the packet was folded over a number times and then a thin piece of wire was pushed through the top corner of the packet. The packet was then plunged into liquid nitrogen (-196 °C) and left in the nitrogen for fifteen minutes. Rapid thawing of the embryos has been shown to be vitally important (Sakai, 1985). This was problematic because some liquid nitrogen infiltrated the packets. On removal from the dewar they swelled up and caused the embryos to lose the contact with the walls of the packet. This problem was solved by tearing a hole in the top of the packet to allow the nitrogen to escape while gentle pressure was applied to the packet for the first few seconds after removal from the liquid nitrogen. After this, the packet was immersed in 30°C water. The feature of this method was that the embryos were kept dry through the whole procedure. Once the embryos had returned to room temperature they were placed on damp filter paper to gently rehydrate them. Morris (1980) reviews this 'dry' freezing technique and points out viability of somatic embryos of *Daucus carota* and immature (hydrated) zygotic embryos of *Zea mays* was completely lost by immediate post-thaw washing, indicating that deplasmolysis is a critical factor in determining survival.

CRYOPROTECTANTS

Two cryoprotectants were used in this project. The first was described by Di Maio and Shillito (1989) and contained 0.5 *M* glycerol, 0.5 *M* L-proline and 1 *M* dimethylsulphoxide (DMSO) at a pH of approximately 5.8. The second cryoprotectant was described by Pence (1991) and contained a final concentration of 0.5 *M* Sucrose and 10% (v/v) DMSO. The first cryoprotectant (Di Maio and Shillito, 1989) was developed for liquid cultures of cells and so the protocol required mixing equal volumes of double strength cryoprotectant with the culture medium containing the cells. In this study single strength cryoprotectant was used. Pence (1991) added the embryos to Murashige and Skoog (1962) medium followed by the addition of double strength cryoprotectant. In this project, culture medium was not used and the embryos were bathed in single strength cryoprotectant.

VIABILITY ASSESSMENT OF INTACT SEEDS

In order to test natural viability of intact seeds, 40 seeds each of *P. elongatus* and *P. falcatus* were planted in vermiculite and placed in the departmental greenhouse. The *P. elongatus* seed were scarified by digging a small hole in the seed near the aryl attachment site using a scalpel blade. In this case the Dithane M-45 was not removed from the seeds. The *P. falcatus* seeds were prepared by removing the hard outer shell and planting the soft internal parts. Here, the Dithane M-45 was on the outer shell of the seeds and so it was discarded along with the shells.

EXPERIMENTAL PROCEDURE AND PRELIMINARY RESULTS

In order to ascertain appropriate culture conditions for the excised embryos a number of experiments were performed. These were: media trials, different light conditions and a test to see if germination could be induced if a small piece of endosperm was left attached to the embryo. The following media were used:

A: MS with 3% sucrose

B: MS with 5% sucrose

C: MS with 1 mg/l of BAP, NAA and GA_3 and 3% sucrose

F: MS with 5 mg/l BAP and 3% sucrose

G: MS with 10 mg/l BAP and 3% sucrose

H: ½ strength MS with 5 mg/l BAP and 3% sucrose

I: ½ strength MS with 10 mg/l BAP and 3% sucrose

J: MS with 0.2 mg/l BAP, 2 mg/l NAA and 3% sucrose

Z: MS with 0.5 mg/l 2,4-D and 5% sucrose

None of the treatments caused embryo germination to occur. The treated embryos neither deteriorated nor grew when they were placed on the culture medium. In order to differentiate between viable, but quiescent and non-viable embryos an experiment was performed which deliberately killed the embryos. These embryos (5 each of *P. elongatus* and *P falcatus*) were placed in 10% sodium dodecyl sulphate (SDS) for 1 hour during which they were sonicated for 15 minutes. The embryos were then surface sterilised with 2% hypochlorite solution, containing a small amount of Triton 100-X (BDH Chemical Ltd.), and put onto a callous inducing medium (MS with 5% sucrose, 0.7% agar and 0.5 mg/l 2,4-D). After some time, these embryos looked no different to the embryos from earlier experiments

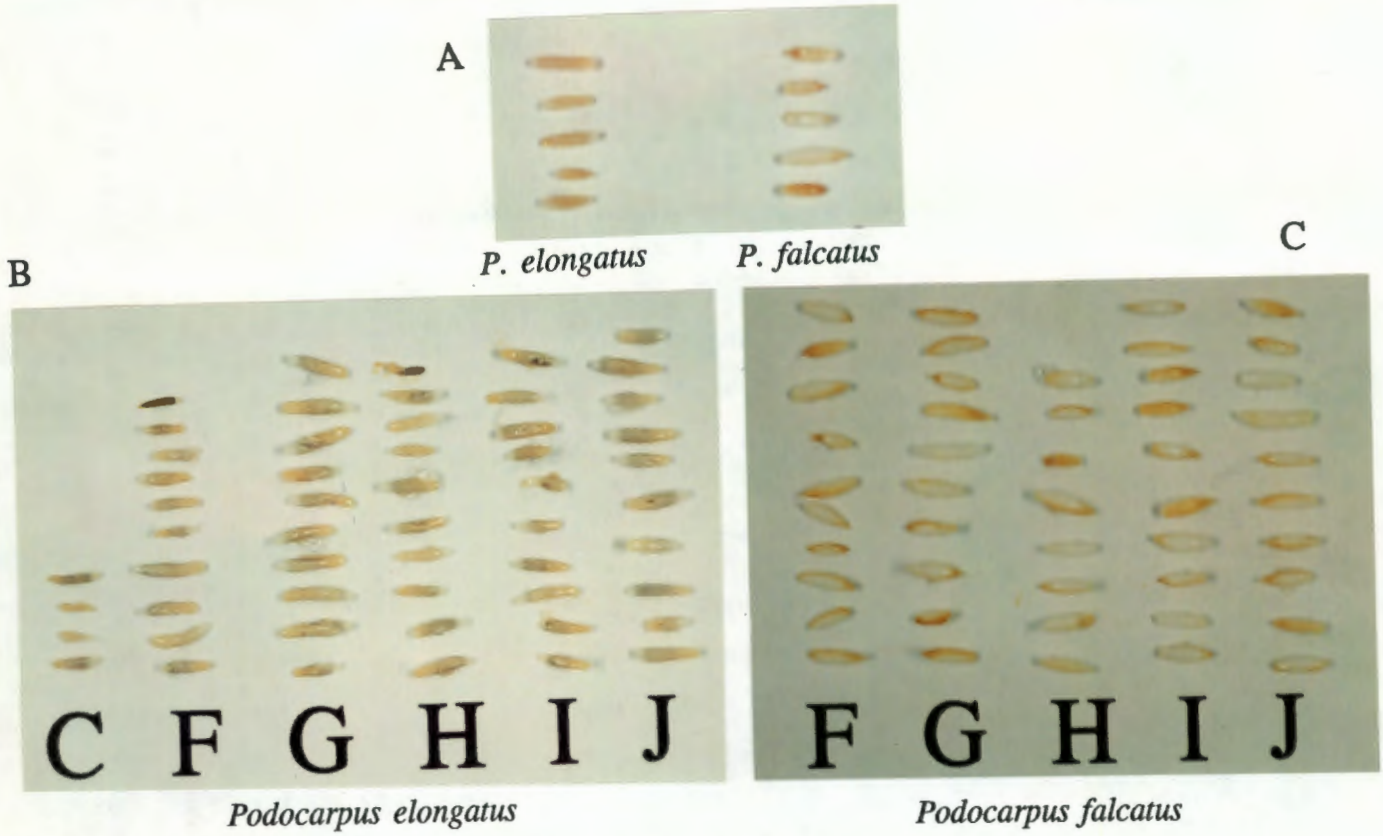

Figure 6: Appearance of the embryos 22 days after sonication and SDS treatment (A), *Podocarpus elongatus* 19 days after the start of the media trial (B) and *Podocarpus falcatus* 19 days after the commencement of the media trial (C). The letters correspond to the media outlined above.

(Figure 6). The outcome of all these preliminary experiments led to the conclusion that the embryos were very sensitive to sterilisation and so a series sterilisation trials were performed on the embryos.

Embryos from the two species were exposed to one of the following treatments with 10% commercial bleach (0.35% hypochlorite) for 5 minutes, 5% commercial bleach (0.175% hypochlorite) for 5 minutes or no exposure to hypochlorite (unsterilised). 4 embryos of each species were used per treatment. Some intact seeds were surface sterilised in undiluted bleach (3.5% hypochlorite) for 10 minutes. The embryos were then aseptically excised. Triton 100-X was not used in any of these treatments. All of the embryos were placed on medium C and put into the 16/8 hour light/dark 25°C growth room.

The results of the surface sterilisation experiment can be seen in Tables 1 and 2. *P. elongatus* embryos gave indications of a positive result after 5 days and so at this point some embryos were used in the test for the presence of chlorophyll, hence the unequal total numbers of embryos. Figure 7 shows the state of the *P. elongatus* embryos after 15 days of development and Figure 8 shows the most prolific callous after a growth period of 43 days.

Table 1: Scores, after 28 days of growth, of the *Podocarpus elongatus* embryos used in the sterilisation test (the values in parenthesis are the scores after 11 days of growth).

Treatment	Number of embryos at each score[1]					Total number of embryos
	++++	+++	++	+	0	
Aseptic excision		1 (1)	4 (1)	(4)	1	6 (6)
5% bleach	3	(3)		2 (2)		5 (5)
10% bleach		2	(2)			2 (2)
Non sterile		3 (2)	(1)			3 (3)

Note

1: The criteria for each score are as follows:

++++	Large, green callous that is growing with clear shoot buds.
+++	Swollen, healthy-looking green callous.
++	Not as large and healthy as +++, greenish brown.
+	Small unhealthy, brown embryo with little callous development.
0	Embryo is small, whitish grey (looks dead)

Table 2: Scores, after 28 days of growth, of the *Podocarpus falcatus* embryos used in the sterilisation test. The callous tissue was colourless, containing no obvious chlorophyll.

Treatment	Number of embryos at each score[1]				Total number of embryos
	+++	++	+	0	
Aseptic excision			1	4	5
5% bleach	1	1		2	4
10% bleach		1	1	2	4
Non sterile	1			3	4

Note

1: The criteria for each score are as follows:

+++	large healthy callous about 5mm in diameter
++	Callous about 3mm in diameter
+	Small callous about 2mm or less in diameter
0	No callous

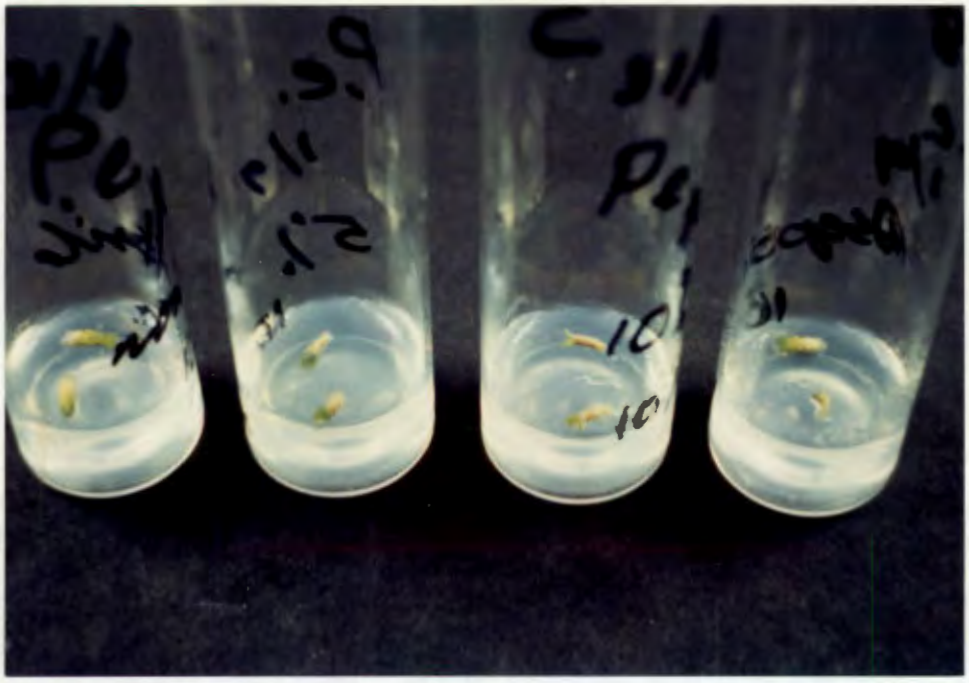

Figure 7: *Podocarpus elongatus* embryos 15 days after the sterilisation test. The treatments are as follows (from left to right): Unsterile, 5% bleach for 5 minutes, 10% bleach for 5 minutes and aseptic excision.

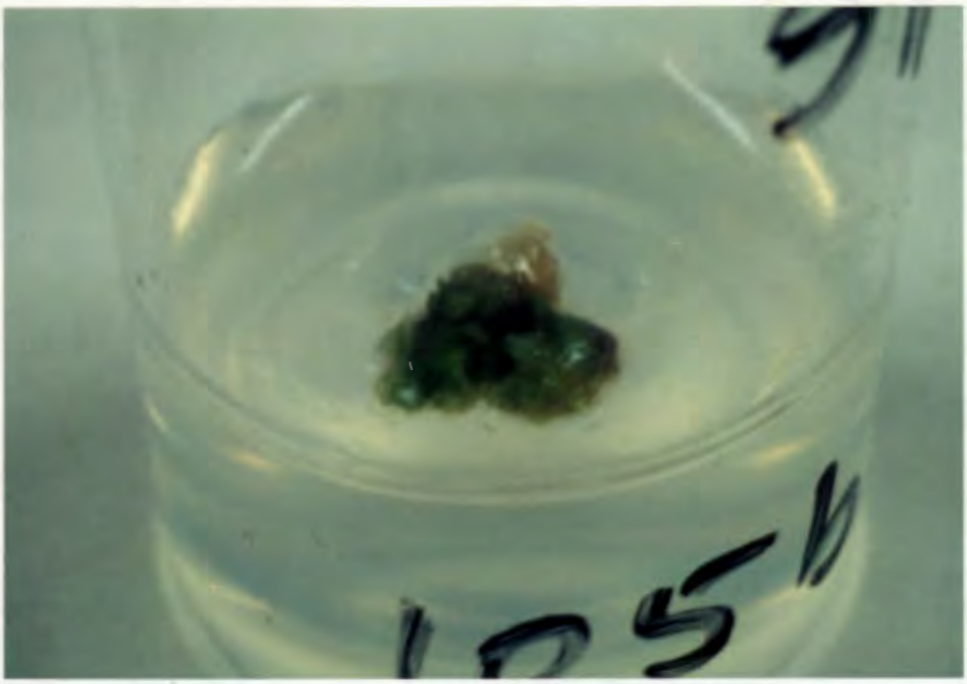

Figure 8: Callous growth, after 43 days, of a *Podocarpus elongatus* embryo treated with 5% bleach for 5 minutes.

A week after the sterilisation treatment, the *P. elongatus* embryos appeared to go green and so they were tested for the presence of chlorophyll by extracting the chlorophyll from individual embryos using 1 ml 80% (v/v) acetone in water. This extract was then scanned using a spectrophotometer and a printout of the absorbances over the wavelengths 400-700nm was obtained. Five *P. elongatus* samples were used:

A green embryo from the unsterilised protocol

A brown embryo from the sterilisation test (10% bleach)

Two yellowish grey embryos from the earlier experiments

Three freshly excised embryos

Some green tissue that attaches the seed to the developing seedling taken from one of the young trees that had grown in the intact germination test.

Two absorbtion spectra from the chlorophyll extraction can be seen in Figure 9. The first one (A) is the spectrum from the control extract from the seedling. In this trace one can see three distinct peaks (I, II and III) which correspond to the standardly accepted peaks of chlorophyll A and B (Lichtenthaler, 1987). The second graph (B) is the absorbtion spectrum from the green embryo. Here one can see very indistinct peaks that could correspond with the peaks seen in A. Unfortunately a replicate scan of the same sample failed to resolve the peaks and so this must be looked upon as a very inconclusive result.

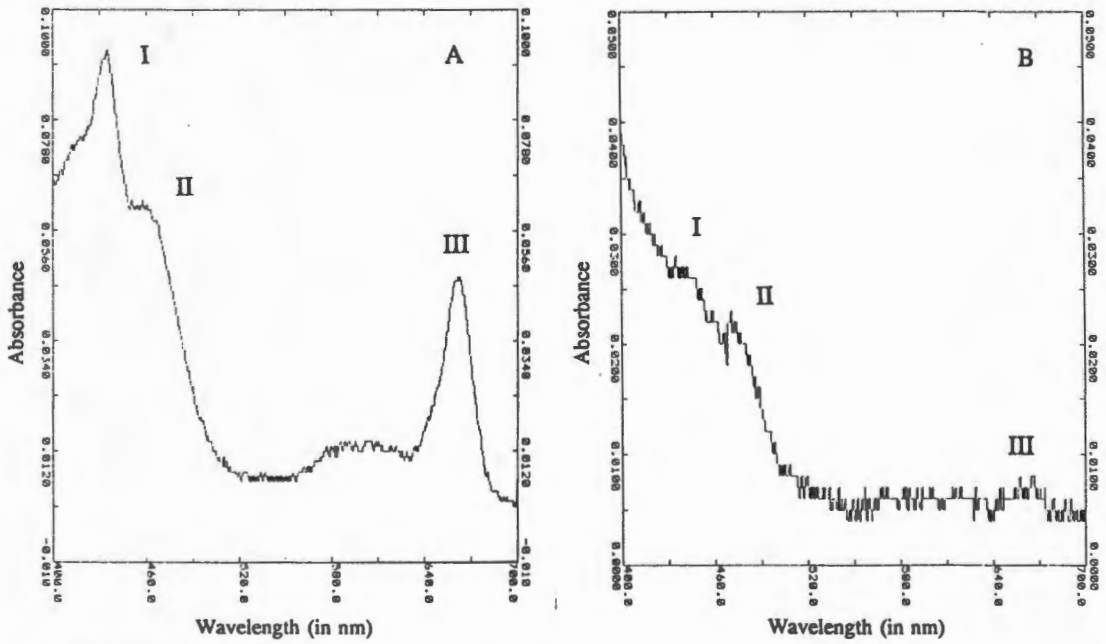

Figure 9: The absorbtion spectra of the chlorophyll extracts of seedling material (A) and a green embryo (B).

(0' control) developed a small patch of bacteria next to it and so I tested this embryo, along with three other non contaminated, 0' control embryos, to see if they could survive surface sterilisation after callousing had commenced. The embryos were placed in 2% hypochlorite for 5 minutes and then thoroughly washed in distilled, autoclaved water.

In all of the experiments survival is defined as the greening of the embryos, accompanied with the development of callous tissue. This criterion of survival has been used before by Chaudhury *et al.* (1991).

Study of the *P falcatus* embryos was stopped because the development of the embryos was too slow for the time available. Both the seeds (see later) and the embryos germinated considerably slower than those of *P elongatus*. Due to the time constraint, it was decided that in the subsequent experiments the embryos would not be sterilised after they had undergone the freezing and drying treatments.

Once the basic culture protocol had been developed the next stage in the project was to study the effect of flash drying and freezing on the *Podocarpus elongatus* embryos.

The first experiment was a drying and freezing experiment performed on *P. elongatus*. Embryos were dried for 0', 10', 20', 40' and 60'. For each drying time 18 embryos were used. Six were used for a moisture determination, six were placed onto medium C to test for viability after drying alone, and six were frozen in the manner described above and then placed onto culture medium C.

Taking the results of the above experiment into account two experiments were performed on *P. elongatus* using the cryoprotectants described earlier. The embryos were dried for 0', 20', 30' and 40'. They were first cryoprotected then the excess liquid was removed using sterile paper towel after which they were placed in the flash drier for the required amount of time. An uncryoprotected and undesiccated control was included in the experiment. Again six embryos were used for the moisture determination, six embryos for viability assessment after drying alone and six embryos were frozen in liquid nitrogen.

The method of cryoprotection using the Di Maio and Shillito (1989) cryoprotectant was as follows. The embryos were added to a vial containing 8 ml of cryoprotectant. The vial was placed on ice for 1.5 hours to allow the cryoprotectant time to penetrate the tissue. After this, the embryos were dried and treated as has been outlined above.

The method of cryoprotection using Pence's (1991) cryoprotectant solution was as follows. 2 ml of water was added to 1 ml of stock (2X) cryoprotectant. The embryos were added to this solution. 15 minutes later 0.5 ml of the stock cryoprotectant was added to the vial. 15 minutes after this, a further 0.5 ml stock cryoprotectant was added to the vial resulting in a final concentration of 0.5 *M* sucrose and 10% DMSO. The embryos were left in this final solution for 1.5 hours after which the were removed from the solution and treated in the manner described above.

A final, brief, experiment was done using some of the embryos that grew in the first successful drying experiment described above. One of the embryos that had not been dried

RESULTS

Seeds of *Podocarpus elongatus* showed the first signs of germination after a period of 19 days. After 84 days 21 seedlings had developed the first whorl of leaves while a further 12 seeds had germinated resulting in a viability of 83%.

Podocarpus falcatus seeds had a very much lower germination rate. 28 days after the seed was planted in the vermiculite there were no signs of growth while 57 days after planting only 3 seeds had germinated.

P. elongatus embryos had an average initial WMB moisture content of 68.9% (DMB of 2.24) while *P. falcatus* exhibited a value of 24.5% (DMB of 0.328).

The results of the drying and freezing experiment are presented in Table 3. It is apparent that survival does not occur at moisture contents of less than about 30%. Initially it seemed that all of the embryos were killed by the freezing treatment but after a period of 34 days it was noticed that one of the embryos had been dried for 20 minutes and then frozen had developed a small amount of callous tissue.

Table 3: Scores, after 21 days of growth, of the *Podocarpus elongatus* embryos used in the flash drying followed by freezing experiment.

Treatment (values are drying times)	Moisture content (WMB %)	Number of embryos at each score[1]				Total number of embryos
		+++	++	+	0	
0'	74.3	6				6
10'	52.6	1	4	1		6
20'	35.2		3	1	3	7
40'	22.1			1	6	7
60'	40.6			1	6	7
0',frozen	74.3				4	4
10',frozen	52.6				4	4
20',frozen	35.2				4	4
40',frozen	22.1				4	4
60',frozen	40.6				3	3

Note

1: The same scoring schedule as used in Table 1 has been used here.

The embryos were not successfully cryopreserved by either of the two cryoprotectants used (see Tables 4 & 5 and Figures 10 & 11). The recovery and growth of the control embryos was also weak and so it is possible that the experiments failed generally and so the result may be unreliable.

Table 4: Scores, after 21 days of growth, of the *Podocarpus elongatus* embryos used in the first cryoprotection experiment using the cryoprotectant described by Di Maio and Shillito (1989)

Treatment (values are drying times)	Moisture content (%)	Number of embryos at each score[1]					Total number of embryos
		+++	++	+	½	0	
Control	70.9	1	2[2]	3			6
0'	61.7			6			6
20'	16.9				5	1	6
30'	23.8				3	3	6
40'	24.8				2	4	6
Control, frozen	70.9					6	6
0', frozen	61.7				1	5	6
20', frozen	16.9					6	6
30', frozen	23.8					6	6
40', frozen	24.8				2	4	6

Notes

1: The same scoring schedule that has been used in Table 1 has been used here. There is on additional category:
½ the embryos are small, brownish and appear sick (between 0 and +).

2: These embryos had calloused but no chlorophyll was present.

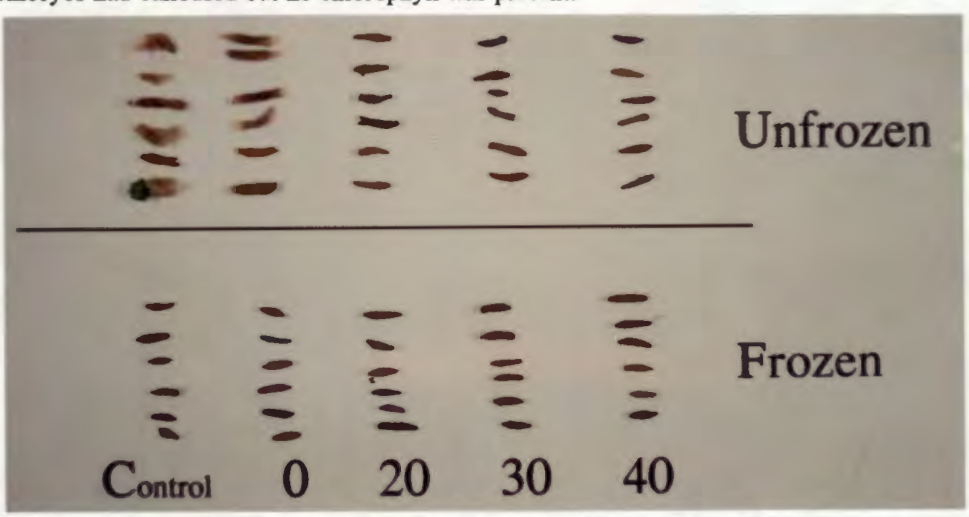

Figure 10: *Podocarpus elongatus* 23 days after the first cryoprotection experiment using the cryoprotectant described by Di Maio and Shillito (1989). Times of drying, after the cryoprotection step, are shown at the bottom while the control group of embryos were neither frozen nor treated with cryoprotectant.

Table 5: Scores, after 20 days of growth, of the *Podocarpus elongatus* embryos from the second cryoprotection experiment using the cryoprotectant described by Pence (1991)

Treatment (values are drying times)	Moisture content (%)	Number of embryos at each score[1]				Total number of embryos
		++	+	½	0	
Control	66.3		3	2	1	6
0'	65.0	3	2		2	7
20'	27.7			3	4	7
30'	24.5			1	1	2
40'	21.0			3	4	7
Control, frozen	66.3				6	6
0', frozen	65.0				6	6
20', frozen	27.7				6	6
30', frozen	24.5			1	3	6
40', frozen	21.0			1	5	6

Notes
1: The same, modified scoring schedule that was used in Table 4 has been used here.

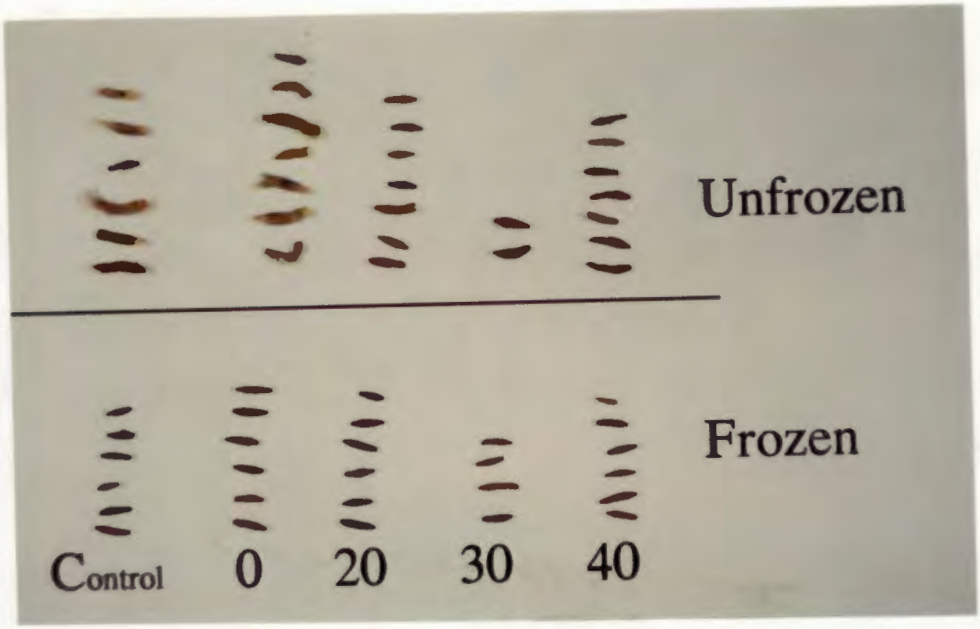

Figure 11: *Podocarpus elongatus* 22 days after the second cryoprotection experiment using the cryoprotectant described by Pence (1991). Times of drying, after the cryoprotection step, are shown at the bottom while the control group of embryos were neither frozen nor treated with cryoprotectant.

Interestingly, the embryos that had been frozen seemed to be more contaminated than those that had just been dried.

The embryos that had been sterilised after commencement of growth seemed to suffer significant die back with the callous tissue going a dark brownish black colour. After a period of 8 days 2 of the embryos were once again developing patches of green tissue on the mound of callous while one of the treated embryos was covered with bacterial growth that resembled the growth that was present near the callous before the sterilisation step. Unfortunately all of the embryos were sterilised concurrently in the same tube and so I cannot say for certain whether this embryo is the one that was initially infected although there is a distinct possibility that this is the case. This indicates that the embryos were not sterilised by the hypochlorite treatment.

DISCUSSION

The most significant result from this study was the discovery that the *Podocarpus* embryos were very sensitive to damage such as that caused by the initial sterilisation technique used (Tables 1 and 2). Damage during the sterilisation steps was also noted by Pritchard and Prendergast (1986) when they surface sterilised embryos of *Araucaria hunsteinii* with 10% commercial bleach. It was found that this treatment resulted in lower fully organised growth than aseptically excised embryos. Direct sterilisation with 0.1% mercuric chloride has been used by some other workers (Chaudhury *et al.*, 1991) although I would be reluctant to work with mercury based compounds.

It is interesting that the unsterilised embryos were predominantly free of infecting organisms. This is in contrast to Mycock and Berjak (1990) who showed that recalcitrant seeds contain a wide range of bacteria and fungi. For example, *Podocarpus henkelii* contained *Fusarium*, *Penicillium* and bacteria within the seed and embryonic axis. A possible reason for the lack of infection is that many embryos appear to be naturally sterile and they only get infected when they come in contact with an unclean environment. An observation supporting is the fact that many workers surface sterilise the intact seed and then aseptically excise the embryos. Using this technique results in sterile embryo cultures. Further support is given by the observation that, in this study, more of the frozen embryos became contaminated than the unfrozen ones. This could be explained by the fact that the frozen embryos were manipulated more that the others and so they had a greater opportunity to become infected. A additional explanation for the elevated infection rate is that the frozen embryos have been significantly damaged and so the embryo's defences have been weakened, making it open to attack from internal pathogenic organisms (Farrant, pers. comm.). In reply to this I would contend that most endophytic bacteria and fungi would thrive on the nutrient rich medium on which the embryos are placed and so I would expect growth irrespective of the health of the embryo. A second reason for the lack of infection could be the presence of antimicrobial agents inside the seed. Seeds are a very rich source of nutrients and so it is necessary for the plant to protect the seeds from all forms of predation, whatever the size of the predator! It would make sense for the plant to deposited antimicrobial compounds inside the seed. Kubo *et al.* (1991) demonstrated the antimicrobial nature of a compound extracted from the root bark of *Podocarpus nagi*. It is possible that such similar compounds are present elsewhere in the tree. If this is the case then support

would be added to the hypothesis that more infection occurs in the frozen embryos because they are no longer capable of producing the antimicrobial chemicals.

THE OPTIMAL MEDIUM

Due to the problem of over sterilisation, the task of finding suitable media and growth conditions for the embryos took considerably longer than anticipated. This meant fewer than expected cryopreservation tests were performed. Other protocols that had been used before were studied and the methods were applied to this new situation. There are two problems with this approach. The most important one is that there have been very few successful cases of seed embryo cryopreservation (reviewed later). The other problem is that the methods used were designed for plants that are completely unrelated to the plants used in the present study and so it is not surprising that some of the methods were unsuitable for this new situation. Simola and Santanen (1990) point out that many of the methods and nutrient media used for conifer tissue culture were developed for angiosperm cultures. This indicates that the media used are probably sub-optimal with respect to growth. If the time had been available, the media trial outlined in the methods section could have been performed again to see if better, organised growth of the treated embryos is possible.

Further, it is important to note that one of the frozen embryos from the drying and freezing experiment performed on *P. elongatus* only started to grow after a period of 34 days after treatment and so it is possible that some of the embryos survived the treatments but were reported as dead because of the severe time constraints. Bajaj (1985) reports that treated embryos can remain dormant for up to 6 months after treatment.

QUICK AND RELIABLE RESULTS

The points made above highlight the problem of getting reliable results as quickly as possible. One solution to this problem is to make use of a conductivity meter (something that is presently unavailable in the laboratory). Here, the experiment on the embryos is performed and then they are placed in the conductivity meter. The meter measures the amount of electrolyte leakage from the embryo and hence the degree of membrane damage. Initially one would have to construct a calibration curve to see what degree of membrane damage is repairable by the cell and what degree of damage results in embryo death. A key advantage of this method is that the embryos can be monitored and then placed into culture

to see what the final outcome of the test is. Such an approach has been successfully used by several authors (for example Pammenter *et al.*, 1991)

Morris (1980) makes some more suggestions concerning rapid tests for viability. For multicellular tissues that have been treated, one can monitor the reduction of tetratzolium salts by the mitochondria. The reduction is quantitative and so the reduced product, formazan, can be assayed spectrophotometrically.

Another possibility involves the intracellular breakdown of fluorescein diacetate to fluorescein which can be visualised in ultra-violet light. The ratio of fluorescent cells to total number of cells gives a quantitative estimate of cellular survival (Morris, 1980). Problems with these methods include the observation that results obtained from the two staining techniques often don't correlate with one another and they both seem to overestimate the actual recovery potential of the cells. Unfortunately the only definitive test is regrowth of the tissue in question (Morris, 1980)

SUBJECTIVITY OF THE RESULTS

A further problem with the results is the subjective manner with which the embryos were scored. Even with a detailed description of the key in mind it was still difficult comparing results that were being taken in the present with those that had been taken earlier. Furthermore each treatment resulted in embryos that needed a different scoring categories, depending on the range of callous development observed, hence the introduction of the ½ category in the two cryoprotection experiments. A way of getting around this problem would be to develop some form of an objective measure of the development of the embryos. The use of laser induced fluorescence of the chlorophyll may be of value. Chappelle *et al.* (1984) shone laser light of 337 nm (ultra violet) onto intact leaves of plants which caused the chlorophyll to fluoresce in the red region of the visible spectrum (between 680 and 700 nm) Spectral quality was shown to be related to the physiological state of the plant being tested. This phenomenon could be utilised to measure the chlorophyll content and health of the embryos. The key advantage of a method such as this is that it is a non invasive technique that could be performed on the embryos whereupon they would be returned to the culture medium to develop further. This method will potentially solve the other significant problem encountered when taking measurements from the embryos. This problem is the very small size of the embryos which was highlighted in the results where it was found that the

chlorophyll extract from the embryos was too dilute to register significantly on the absorbtion spectra (Figure 9B).

EFFECT OF THE CRYOPROTECTANTS

Although no conclusive results were obtained there is some empirical evidence that the cryoprotective solutions used in this study did actually help to reduce the freezing injury of the embryos. It has been assumed that the embryos were bathed in the cryoprotectant solutions for long enough to allow the protectant time to penetrate the tissue. Morris (1980) makes the valid point that upon thawing, cryoprotective additives are potentially cytotoxic. DMSO inhibits cellular metabolism and so Morris (1980) states that this chemical must be effectively removed from the tissue. In this experiment the embryos were not rinsed and so it is possible that the germination of the embryos was inhibited by the presence of the DMSO. The only removal of DMSO that could have occurred was diffusion into the moist filter paper or the growth medium.

IS *PODOCARPUS ELONGATUS* RECALCITRANT?

Berjak *et al.* (1989) propose working definition of recalcitrant seeds which is: seeds are recalcitrant if they are shed wet and cannot be dehydrated or stored. In this project it was found that *P. elongatus* embryos exhibited a high moisture content of close to 70%. It is indicated that the embryos can only be flash dried to approximately 30% moisture content before they lose viability. These two pieces of evidence, along with the intolerance of freezing, lend support to the hypothesis that *Podocarpus elongatus* trees produce reasonably recalcitrant seeds. In order to verify this, a much more rigorous study examining this question would have to be performed. If this species is recalcitrant, then results of the present study can be compared to those from other recalcitrant species.

REVIEW OF OTHER STUDIES CONCERNED WITH EMBRYO CRYOPRESERVATION

As I have mentioned before there are very few successful instances of cryopreservation of recalcitrant seed embryos. In this section I will briefly review what other workers have done in the field of recalcitrant seed cryopreservation.

Initially it was thought that oil palm seed (*Elaesis guinensis*) was recalcitrant (Chin and Roberts, 1980) but, on reevaluation, it was suggested that they exhibited orthodox

behaviour (Grout *et al.*, 1983). It was shown that intact seeds could not be cryopreserved but it was possible to store the embryos for 8 months at -196°C with no apparent loss in viability. The embryos were not cryoprotected, they were only desiccated to some extent before they were plunged into the liquid nitrogen. The embryos were aseptically excised from surface sterilised seeds.

Pritchard and Prendergast (1986) found that optimal survival of cryopreserved *Araucaria hunsteinii* embryos occurred at an intermediate moisture content of 20%, indicating that there is a trade off between desiccation damage and freezing damage. Unfortunately no data are presented that show reduced survival after freezing for embryos with a moisture content of higher than 20% and so these result should be viewed with some circumspection. Again no cryoprotectant agents were used, the embryos were just placed in polypropylene ampoules that were placed directly into the liquid nitrogen. Two different sterilisation treatments were performed. The embryos were either aseptically excised from sterilised seed or directly surface sterilised with a 10% solution of commercial bleach for 20 minutes. It was found that the aseptically excised embryos exhibited more fully organised growth than the directly sterilised embryos.

Eighteen species of large seeded, temperate trees were tested for their tolerance to cryopreservation in liquid nitrogen (Pence, 1990). The excised embryos were surface sterilised with 5% commercial bleach for 10 minutes followed by two washes with sterile water. Again no cryoprotectant agents were used, the embryos were just dried overnight on the laminar flow bench and then immersed in the liquid nitrogen. The moisture content of the different species' embryos ranged from 10% down to 5.6%. Survival ranged from very good and normal in appearance to poor with only limited callous growth. It was also shown that fresher seed was more tolerant to the treatments than older seed.

In the another study, Pence (1991) found that mature cacao (*Theobroma cacao*) embryos were intolerant to hydrated freezing or desiccation and so it was decided to examine the survival of the immature embryos because they are smaller and so would freeze and thaw a lot quicker as well as having a greater embryogenic capacity. The embryos were removed aseptically from sterilised seeds. This experiment was technologically much more advanced, the embryos were precultured on concentrated sucrose medium, cryoprotected using the solution described earlier in this text and they were frozen by being cooled at 0.4°C per minute until a temperature of -35 to -40°C was achieved. After this, the embryos were

slowly thawed or immersed in liquid nitrogen overnight. Rather poor results were achieved with the best being callous growth and somatic embryogenesis occurring in the embryos that had been stored overnight in liquid nitrogen.

Chaudhury *et al.* (1991) managed to cryopreserve embryonic axes from tea (*Camellia sinensis*). It was shown earlier that intact seeds could not be desiccated to below 20% moisture content and they were sensitive to freezing injury. In this study the only pretreatment was desiccation in a sterile flow of air before the axes were frozen by immersion into liquid nitrogen. The embryos survived if they were dried down to a moisture content of 13% or lower. Clearly some cellular damage occurred because the treated embryos could not survive as long as the untreated embryos on moist filter paper. They had to be cultured on a nutrient medium to ensure survival. Wesley-Smith *et al.* (1992) also managed to achieve successful cryopreservation of tea embryos. The excised embryos were flash dried before being frozen. In this study Wesley-Smith *et al.* (1992) found that there was a very narrow window of survival flanked by desiccation damage on the one side and freezing damage caused by intracellular ice crystals on the other.

The final study reviewed here is one performed by Radhamani and Chandel (1992). As with Chaudhury *et al.* (1991), the embryos were aseptically excised from sterile seed and the only pretreatment prior to freezing was desiccation in a sterile flow of air. This study clearly showed that there was an optimum degree of drying (14%) to get maximally successful freezing. Above and below this value fewer embryos survived the treatment. It was also shown that storage at -197°C for a period of eight months resulted in embryo viability that was equal to that of the embryos that had been frozen overnight.

FUTURE STUDY

This project is essentially a preliminary study that should act as a platform from which a more detailed study should be performed. Experiments that should be done include the following.

A storage profile of the intact seeds needs to be performed to see if the storage at 10°C has any influence on the seed viability. Demonstration of viability deterioration would significantly affect the results presented in this project. This information could also be used in deciding the degree of recalcitrance of the seeds.

A media trial should be performed using the new, mild sterilisation technique to see if the embryos can be induced to grow in an organised manner as opposed to the callous development achieved up to this point. If better growth is achieved, then the drying and freezing experiments need to be repeated in order to ascertain whether there is any change in the tolerance of embryos due to the more suitable growth medium.

In order to find optimal conditions, a number of different rates of freezing should be tried. An example, is the freezing protocol suggested by Merryman and Williams (1985). Here the embryos would be held at a constant temperature of about -30°C for some time after which they would be transferred to liquid nitrogen. The initial low temperature allows for the development of extracellular ice which would have the effect of dehydrating the cells and so they are more tolerant to the subsequent deep freezing.

One could also experiment with different concentrations and compositions of cryoprotectant solutions. Although highly inconclusive there are indications that the first cryoprotectant used, Di Maio and Shillito (1989), severely inhibited the subsequent growth of the embryos (see Table 4, compare the control with the t = 0 minutes). It has been shown that trehalose is an extremely effective cryoprotectant that works by acting as a substitute for water in the stabilisation of membranes (Bhandal *et al.*, 1985). It would be worthwhile to see if favourable results could be achieved using this cryoprotectant.

Most importantly once a positive result has been obtained the experiment must be repeated a number of times the ensure that the result is normal and not anomalous.

ACKNOWLEDGEMENTS

A sincere thankyou needs to go to the following:

Dr Jill Farrant for her patient tolerance of my experimental style and her skilful guidance in the production of this report.

The Flora Conservation Committee of the Botanical Society of South Africa for financial support.

The Kirstenbosch Seedroom for donating the *Podocarpus elongatus* seeds used in this study.

The members of lab 3.01 for taking the tedium out of repetitive laboratory work.

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