THE DEVELOPMENT OF PROTOCOLS FOR THE DIAGNOSIS AND MICROPROPAGATION OF COLD-TOLERANT *EUCALYPTUS* CULTIVARS

by

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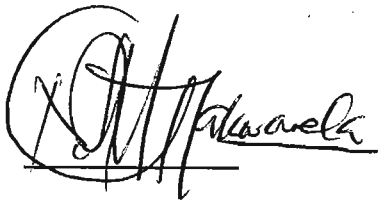
PREFACE

The experimental work described in this thesis was carried out in the Department of Biology, University of Natal, Durban, from January 1995 to December 1996, under the supervision of Dr. Paula Watt.

These studies represent original work by the author and have not been submitted in any form to another university. Where use was made of the work of others it has been duly acknowledged in the text.



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Best for last, thanks to God Almighty who made sure I survived to tell the tale.

JOB 34:13,2-4.

"[2]Hear my words, you wise men;

Listen to me, you men of learning.

[3]For the ear tests words as the

tongue tastes food.

[4]Let us discern for ourselves

what is right; Let us learn

together what is good."

ABSTRACT

In South Africa, *Eucalyptus* trees are used for many processed wood products (e.g. paper) and in the mining industry. Priorities in *Eucalyptus* breeding programmes include selection of varieties that are fast growers, insect and disease resistant, have appropriate wood characteristics and can grow in a wide variety of environmental conditions. Cold-tolerant cultivars of *E. saligna* and of *E. grandis* have been bred and selected in Australia and South Africa, respectively for use in cold regions of Natal Midlands and North Eastern Cape. However, the production of large numbers of such cultivars for planting out in a commercial scale is being impaired by slow growth rate, low regeneration time and poor rooting ability of cuttings from these trees. Consequently, methods of *in vitro* propagation of cold-tolerant clones were investigated.

Axillary buds were induced and subjected to a variety of multiplication, elongation and rooting media. The optimised protocol for the production of shoots from axillary buds was: bud induction medium comprising of MS supplemented with 20 gl^{-1} sucrose and 10 gl^{-1} agar for 1-2 weeks, multiplication medium comprising of MS supplemented with 0.1 mgl^{-1} biotin, 0.1 mgl^{-1} calcium pantothenate, 0.2 mgl^{-1} benzyladenine phosphate, 20 gl^{-1} sucrose and 3.5 gl^{-1} Gelrite for 4 weeks, elongation medium for 4-6 weeks comprising of MS medium supplemented with 0.1 mgl^{-1} biotin, 0.1 mgl^{-1} calcium pantothenate, 0.35 mgl^{-1} NAA, 0.1 mgl^{-1} kinetin, 0.1 mgl^{-1} IBA, 20 gl^{-1} sucrose and 3.5 gl^{-1} Gelrite. Production of plantlets via somatic embryogenesis was also investigated but hampered because of high rates of contamination as pieces of mature leaves were used as explants.

Ongoing breeding programmes are aimed at obtaining hybrids of *Eucalyptus* that are cold-tolerant. The hybrid progeny then need to be screened for cold-tolerance. However, a major problem in the selection of cold-tolerant clones is that diagnosis can only be undertaken by

assessing the field performance of the genotypes under various environmental conditions. In this regard, a protocol for 1D gel electrophoresis was developed for *Eucalyptus* species with the view to use it for the detection of cold-tolerant stress proteins. Leaf material from both non-cold tolerant and cold-tolerant clones was used. Well-resolved gels that focused on the comparison between protein profiles of cold-susceptible and cold-tolerant clones before and after period of cold stress were obtained. The findings of this study showed that two polypeptides, one in the lower molecular region of 14.3-20.1kD and another of a higher molecular weight in the region of 116.4-170 kD were observed after cold acclimation. These changes in polypeptide profiles were observed in cold-tolerant *E. grandis* x *nitens* (GN1) and *E. saligna* (AS 184, AS 196 and TS 15) but not in a non-cold tolerant species *E. grandis* (TAG 731). These polypeptides may have an important role in the cellular adaptation to cold temperatures. It is suggested that this method may be used as a diagnostic tool for screening cold tolerance on *Eucalyptus* cultivars.

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LIST OF ABBREVIATIONS

%	percent
%T	polyacrylamide gel concentration defined as percentage total monomers (i.e. acrylamide + crosslinking agent, g/100 ml)
µ	micro
$\mu E.m^2.s^{-1}$	micro Einsteins per metre squared per second
µl	microlitre
°C	degree Celcius
2,4-D	2,4-dichlorophenoxyacetic acid
ABA	abscisic acid
BA/BAP	6-benzylaminopurine
BSA	bovine serum albumin
CIM	callus induction medium
cm	centimetre
DMSO	dimethylsulphoxide
EDTA	ethylene diaminotetracetic acid
fmass	fresh mass
g	gram
g	gravitational force
GA_3	gibberellic acid
h	hour
ha	hectares
IAA	indole-3-acetic acid
IBA	indole-3-butyric acid
l	litre
M	molar (mole. l^{-1})
mA	milliamps
mg	milligram
min.	minutes
ml	millilitre
mm	metre
mM	millimolar
mmol	millimole
MS	Murashige and Skoog (1962) nutrient formulation
MW	molecular weight
n	number of observation
NAA	1-napthylacetic acid

PAGE	polyacrylamide gel electrophoresis
pH	hydrogen ion concentration
PMSF	phenylmethylsulphonyl fluoride
PPFD	photosynthetic photon flux density
PVP	polyvinyl pyrrolidone
s	second
SDS	sodium dodecyl sulphate
SDS-PAGE	polyacrylamide gel electrophoresis in the presence of SDS
spp.	species (plural)
TCA	trichloroacetic acid
TEMED	N,N,N',N'-tetramethylethylenediamine
T_{max}	gel concentration for maximum separation between two proteins
Tween 20	polyoxyethylene sorbitan monolaurate
V	volts
v/v	volume per volume
w/v	weight per volume

CHAPTER 1

INTRODUCTION

1.1 Forestry in South Africa

From a country relatively poorly endowed with natural forests and timber resources, South Africa has grown into a world leader in plantation forestry (Anon., 1996a). It all started with a pioneering plantation in the Western Cape established in 1876 to supplement the sparse natural sources of fuel for the early railroads reaching into the interior (van der Zel, 1989; Anon., 1996b). After the culmination of many years of growth and development, especially in the wake of World War II, today plantations cover 1.3 million hectares, 1 percent of the country's total surface area (Dept. Water Affairs and Forestry, 1996). Their distribution is shown in Table 1.1. Advanced tree breeding and selection has led to the development of superior trees, that grow as much as 15 mm per day and increase the timber yield substantially. The South African forestry industry is capable of meeting the bulk of the timber needs of the local population and of the industry (van der Zel, 1988). The commercial forests represent a complex and valuable resource yielding many different products such as sawlogs, poles, pulpwood, mining timber, tan bark and veneer logs (Anon., 1996b). Timber is grown in plantations of exotic species in areas with adequate rainfall and suitable soil. Certain categories of wood and wood-based products such as high quality furniture timbers, art papers and certain blending pulps are still imported, but in net terms South Africa is also an exporter of wood products (van der Zel, 1988).

Prior to 1938 Southern Africa's silvicultural and management systems were based on those of Europe, but gradually it became apparent that the man-made forests with all the costs pertaining thereto, could not be compared economically and silviculturally with the natural forests in other parts of the world (van der Zel, 1989). Based on research by Craib, the Department of Forestry of the Republic of South Africa completely departed from the established methods. Planting widths were increased from a maximum of 1.8 m to 2.7 m and the thinnings policy was changed from light thinnings to heavy numerical thinnings, reducing the rotation from 40 and 60 years to 30 and 40 years for quick and slow-growing pines, respectively (van der Zel, 1989). The object of management of most eucalypt plantations is the production of pulpwood and mining timber on short rotations in which case the need for the thinning does not arise (Schönau and Stubbings, 1987). Thinnings are applicable only when eucalypts are grown for the production of saw or veneer logs, or heavy tramission poles. In general, the first thinning should take place early and be heavy enough to eliminate suppressed trees and those of poor form.

1.2 Brief history of the use of *Eucalyptus* in South Africa

The genus *Eucalyptus* is of great importance to forestry since it provides many species which are used for commercial plantations throughout the world. The planting of *Eucalyptus* for commercial wood production in South Africa began more than a century ago (Poynton, 1979; Darrow, 1994). With the development of the Transvaal gold mines there arose a great demand for mining timber which could be quickly and cheaply produced (Read, 1929; Darrow, 1994). Limited to the Cape Province at first, the planting of eucalypts spread to the Transvaal both before and particularly after the Anglo-Boer War of 1899 to 1901 (Darrow, 1994). However, the early history of the

introduction of the cold-tolerant *Eucalyptus* species to the Transvaal is not well known (Darrow, 1984).

Table 1.1: Plantation area of commercial forest species by Province/Ownership Adopted from Plantation Statistics, 1994/1995 (Dept. Water Affairs and Forestry, 1996).

Province	Ownership 1994/1995 (Hectares)			Privately owned (%)	Publicly owned (%)
	Private	Public	Total		
Northern Province	38. 915	24.955	63.870	60.9	39.1
Mpumalanga	445.982	143.336	589.318	75.7	24.3
North West Province	0	0	0	0.0	0.0
Gauteng	0	0	0	0.0	0.0
Free State	100	8	108	92.6	7.4
Kwazulu/ Natal	452.092	79.768	531.860	85.0	15.0
Eastern Cape	33.541	127.471	161.012	20.8	79.2
Northern Cape	0	0	0	0.0	0.0
Western Cape	23.901	58.561	82.462	29.0	71.0
Total R.S.A	994.531	434.099	1.428.630	69.6	30.4

In about 1929, investigations by the Forest Products Institute of the Department of Forestry showed that timber of *E. saligna* was suitable for purposes other than mining timber e.g. for box shocks, parquet flooring, office and other furniture. This led to an increased demand for logs of dimensions greater than those required by mining industry (van Laar, 1961). The years that followed witnessed further changes, e.g. the development of the Orange Free State gold fields resulting in a further increase in the demand for mining timber (van Laar, 1961).

The first mention of the selection of different *Eucalyptus* species for frost tolerance was by Beard (1958) who visited Australia to determine what species would be suitable for afforestation work in the Eastern Transvaal Highveld (ETH). That author mentioned that among the cold-tolerant *Eucalyptus* species already growing satisfactorily in the ETH were *E. macarthurii*, *E. dalrympleana*, *E. andreana* and *E. nitens*. Other species known for their good growth rates but with less frost resistance were *E. obliqua*, *E. fastigata* and *E. fraxinoides* (Darrow, 1994). There has been generally scanty information published about cold-tolerance and growth and among the hard gums in South Africa before the late 1970s (WRI, 1977). Bearing in mind the paucity of the information, in 1990 the Institute for Commercial Forestry Research (ICFR) began a series of trials planted in three zones namely: ETH, Natal Highlands, and North Eastern Cape, above 1400 m altitude with the specific aim of comparing a wide range of selected *Eucalyptus* species, known to be moderately to highly cold-tolerant, in areas that were cold and relatively dry. The results of that study found that those species that survived the frosts of the higher altitudes in the summer rainfall areas of South Africa, while at the same time showing excellent height growth, were the riverine species. Those were a group of species , *viz. E. macarthurii*, *E. badjensis*, *E. viminalis*, *E. benthamii* and *E. smithii*, which are all closely related (Pryor and Johnson, 1971) and regarded as part of the subseries *Viminalinae*. They are all described as occurring on alluvial soils or in valley bottoms (Blakely, 1965, Darrow, 1994). Using Nixon's (1983) study, Darrow (1994) ranked the species for frost hardiness and survival in the following order: *E. regnans* < *E. delegatensis* < *E. globulus* var *maidenii* < E. *globulus* var *biscostata* < *E. elata* < *E. fastigata* < *E. nitens* < *E. viminalis* < *E. macarthurii*. Another report (SAPPI, 1992) concerning the frost

resistance of 90 seedlots of nine *Eucalyptus* species at nine months of age (after one winter), planted at Dorstbult in ETH ranked the species for decreasing frost tolerance as: *E. oreades* < *E. fraxinoides* < *E. saligna* < *E. grandis* < *E. nitens* < *E. macarthurii*.

1.3 Damage to *Eucalyptus* species by low temperature stress

It is of fundamental importance to understand the nature of stress injury and resistance prior to the examination and discussion of cold-tolerance in the present study. *Eucalyptus* species usually manifest frost damage within a few days of frosting, whether subjected to artificial (Harwood, 1980) or natural frosts (Paton, 1972). The most noticeable is foliage damage usually characterized by a "coumarin-like" odour and the development of a flaccid, water-soaked appearance which gives way to necrosis and death (Paton, 1972; Tibitts, 1986). Depending on the severity of frost, damage may be confined to small patches on leaves remaining for some time (Bond, 1945) or falling, as usually is the case (Paton, 1983). Severe damage may kill all foliage eventuating in complete defoliation (Fig. 1.1). Buds and stems are also killed by frost with slower visual expression of damage than in leaves (Ashton, 1958). Natural populations of *Eucalyptus* species are occasionally damaged to varying extents following unusually severe frosts, and in some instances broad scale death of large trees and large areas of forest have been reported (Tibbits, 1986). However, severe frosts are known kill to stems to ground or the whole tree level (Pilipenka, 1969). Unusually severe winter frosts caused the death of large areas of six-year-old *E. nitens* and *E. delegatensis* regeneration on the Toorongo Plateau of Victoria (Bond, 1945). Frost damage to planted *Eucalyptus* has been reported in countries such as South Africa (Nixon, 1977; 1983; Darrow, 1984), Zimbabwe (Quaile and Mullin,

1983), Argentina (Mendonza and Alliani, 1983), New Zealand (Rook *et al.*, 1980; Wilcox, 1982a,b,c) and France (Francelet and Boulay, 1982). The quantification of a tree's response to freeze damage can be difficult. Theory and practice have led to an adoption of a meaningful scoring system. Figure 1.1 is a quantification system of damage sustained to crown and stem.

1.4 Modes of action by cold temperature stresses

In areas where climate varies between a cold and a warm season, plants tend to develop different ways of surviving the unfavourable season (Skre, 1988). Plants, where overwintering buds, bark and needles have developed a high tolerance against low temperature, therefore have an advantage. During the year plants may be subjected to many different types of damage depending on season, stage of development and climatic conditions. Of special importance is the rate of freezing and thawing. Rapid temperature drops may cause supercooling and intracellular freezing and subsequent irreversible membrane rupture within the cells (Skre, 1988). The basic structure and organisation of most plant tissues and cells dictate to a large extent the site of initiation of ice crystallization, determining the course and location of cell-water freezing (Burke *et al.*, 1976). The freezing process in plant tissues is affected by the following facts: a) plant cells are surrounded by a water saturated environment, b) the amount of osmotically available extracellular water is small relative to the amount of water inside the living cell, c) the apoplastic solution has a much lower solute concentration than the cell, d) cellular water, because of the higher solute concentration , has a greater freezing point depression (Levitt, 1972; Levitt, 1980), and e) the effectiveness of a cell membrane as a barrier to ice may vary with

cold acclimation or temperature (Dowgert and Steponkus, 1984). The other major factor in the freezing of plant cells is the rate of cooling (Guy, 1990).

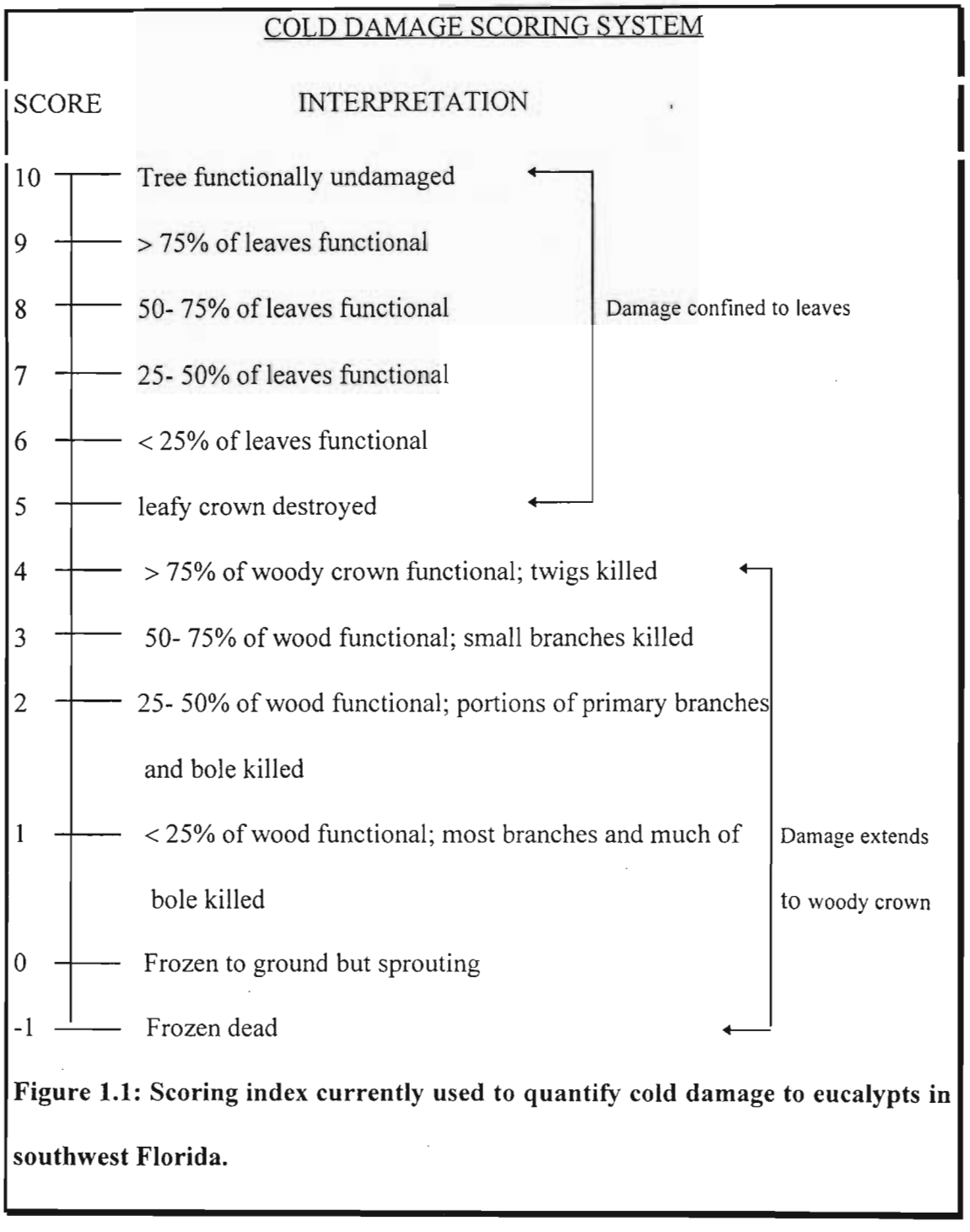


Figure 1.1: Scoring index currently used to quantify cold damage to eucalypts in southwest Florida.

1.4.1 Intracellular freezing

Depending on the rate of tissue cooling, the crystallization of ice can occur in two markedly distinct locations within the tissues of most plants (Guy, 1990). If cooling is rapid, ice may form within the cells. Crystallization of the water inside the cell may occur by internal nucleation (Levitt , 1956) or by penetration into the cell by an external ice crystals. In either case such freezing, termed intracellular freezing, is considered to be universally and instantaneously lethal (Burke *et al.*, 1976; Levitt, 1972; 1980). Asahina (1956) found two kinds of intracellular freezing. In hardened cells the vacuole remained unfrozen, and the freezing rates were slow and in unhardened cells the different cell compartments froze simultaneously. In intracellular freezing the damage is mostly mechanical by membrane rupture, while low molecular weight proteins and free nucleic acids remain intact (Skre, 1988). Some circumstantial evidence suggests cooling rates less or equal to 3 °C h^{-1} may favour intracellular freezing in intact plants: fortunately in nature, atmospheric cooling rates seldom exceed 1 °C h^{-1} (Guy, 1990).

1.4.2 Extracellular freezing

During extracellular freezing, the cooling rate is slow enough to allow water to diffuse out of the cells into the intracellular space, where vapour is lower due to the presence of more aerosol particles, and where ice crystals may be formed (Levitt, 1956; Skre, 1988). By ordinary extracellular freezing, White and Weiser (1964) found that the tolerance limit at mid-winter in thuja leaves was 20- 40 °C below the actual mean minimum temperatures. According to Asahina (1978) water diffusion from the cell into the intercellular space is caused by the different water potential between

supercooled water inside the cell and ice outside. Many theories have tried to explain the modes of action during extracellular freezing. Lovelock (1957) and Meryman (1960) constructed the salt damage hypothesis, i.e. when cells are dehydrated, the protein from lipid-protein complexes precipitated because of high concentration of electrolytes in the cells. Santarius (1969) showed that increased sodium chloride concentration caused faster damage in isolated spinach chloroplasts by uncoupling of adenosine triphosphate (ATP) synthesis from electron transport relative to unfrozen control samples. The damage occurred partly by removal of structural water and partly by increasing salt concentration. Water binding additives like glycerol, sugar and polyvinyl pyrrholidon (PVP) could prevent the damage (Meryman, 1960; Skre, 1988).

1.5 Genetics of freezing tolerance

1.5.1 Overwintering

Freezing tolerance in temperate biennials and perennials, is the phenomenon that enables them to survive winter conditions and resume growth and development in the spring (Guy, 1990). This is not simply a function of the minimum survivable temperature (LT_{50}) but involves a genetically programmed, integrated process (Guy, 1990). To survive the winter, a plant must have evolved mechanisms whereby freezing-sensitive tissues can avoid freezing or undergo a change in freezing tolerance compatible with the normal variations of the local climate. This involves coordinate of the induction of the tolerance at the appropriate time, and maintenance and resumption of growth when the risk of freezing has passed. Since, overwintering or winterhardiness is an important agronomic trait in many crops, most studies have

focused on cultivar and varietal trials (Fowler and Gusta, 1979; Cain and Andersen, 1980), and selection of cold-tolerant lines (Hoard and Crosbie, 1985), with less attention given to the quantitative genetics (Gullord *et al.*, 1975; Hummel *et al.*, 1982; Limin and Fowler, 1988, Guy, 1990). Breeders have exploited genetic variability present in many crops to develop cultivars with improved cold tolerance, while geneticists have used natural variation to examine inheritance (Guy, 1990). Crosses involving compatible parents of differing cold tolerance typically yield progeny exhibiting a continuous range of hardiness between the parental extremes. Since this has been the most common observation for progeny of such crosses, it has been concluded that winterhardiness is a quantitatively inherited trait (Limin and Fowler, 1988).

1.5.2 Cold acclimation

A significant component of winterhardiness in cold-hardy plants is the capacity to undergo cold acclimation. Virtually all temperate perennial, and many annual and biennial plants native to the regions of the world subject to subzero temperatures can alter their tissue and cellular freezing tolerance upon exposure to low non-freezing temperature (Levitt, 1972, 1980; Guy, 1990). The term cold acclimation is most often used to describe the outcome of the myriad biochemical and physiological processes associated with the increase in cold tolerance. A more precise view of cold acclimation would include two major functions, the more universal adjustment of metabolism and basic cellular function to the biophysical constraints imposed by LT, and the induction of freezing tolerance. The first function of cold acclimation differentiates chilling-sensitive from chilling-tolerant species while the second

function discriminates chilling-tolerant but freezing-sensitive species from those that are freezing tolerant (Guy, 1990). Guy (1990) further indicated that presently it is not known if both functions are required for the development of freezing tolerance at LT. Certainly if a plant cannot adjust cellular processes for proper function during long-term exposure to low non-freezing temperatures, it is unlikely to become maximally freezing tolerant. Nearly all genetic studies of cold hardiness have focused on aspects other than cold acclimation, primarily overwintering ability (Law and Jenkins, 1970; Cahalan and Law, 1979; Limin and Fowler, 1988; Guy, 1990). Exogenous abscisic acid (ABA) treatment of hardy species has been shown to induce freezing tolerance at normally nonacclimating temperatures (Zeevaart and Creelman, 1988; Lång *et al.*, 1989; Robertson *et al.*, 1995).

1.6 Clonal forestry of *Eucalyptus* species

A development of the last decade is the bringing to practical reality clonal culture of *Eucalyptus*. The vast areas of South Africa are characterized by a dry environment and only a portion of the country has good rainfall, fertile soils and climates favourable for tree growth (Denison and Kietzka, 1993a). Such areas are scarce and highly in demand for the planting of agricultural crops and breeding of livestock. As a result, eucalypts are grown largely on marginal land sites in the southern Cape, Natal Midlands, eastern and north-eastern Transvaal (Denison and Kietzka, 1993a). The eucalypts selected for growth in these areas need to be adapted to the environmental and climatic conditions of the site, show good growth performance, be tolerant to diseases and pests, and produce wood of high yield and quality for

processing (Denison and Kietza, 1993b) and such species have been obtained through clonal breeding programmes.

Denison and Quaile (1987) planted clonal trials in South Africa to test variability in growth parameters. Sixty clones of *Eucalyptus* were included in the trial, and at two years showed statistically significant differences in height and volume growth. The mean annual increaments of the top-five ranked clones (not named) was 17.2 m^3ha^{-1} and 18.7 m^3ha^{-1} at 24 months. A noticeable characteristic of this trial was the uniformity of growth within plots of a given clone which was statistically expressed in the low coeffiecients of variation calculated. Those authors also anticipitated the uniformity within a clone to be also evident in the wood properties. The effect of fertilizers was also tested in combination with burn and non-burn treatments. The results showed that burning stimulated initial height growth and improved uniformity.

Clonal forestry has become very important and exclusively practised in South Africa. Micropropagation by axillary bud proliferation could aid clonal programmes as it encompasses all the advantages of vegetative propagation and, in addition, it allows for greater yields in a shorter period of time than macropropagation (i.e. via cuttings). Micropropagation offers advantages over traditional methods because cultures are started with very small pieces of explants, and thereafter small shoots or embryos are propagated. Only a small amount of space is required to maintain plants or to greatly increase their number hence resulting in mass propagation. Plants may acquire a new temporary characteristic through micropropagation which makes them more desirable to the grower than conventionally-raised stock. Production can be continued all the

year round and is independent of seasonal changes. Traditionally, clonal programmes make use of cuttings from a parent/selected plant that are then rooted to produce a new plant/clone.

1.7 Aims of this investigation

To meet the estimated future demands for hardwood in South Africa, planting of eucalypts has now extended to areas which are subjected to low temperature. Towards this end the industry is developing programmes for the selection and clonal multiplication of cold-tolerant genotypes. As mentioned, tissue culture can aid such programmes. However, at present, there are no methods available for micropropagation of cold-tolerant clones. Consequently, in this study, methods of *in vitro* propagation of cold -tolerant clones via axillary bud proliferation and indirect somatic embryogenesis were investigated. In addition to trying to multiply known cold-tolerant clones, the industry is also interested in identifying others that could be cold-tolerant. Traditional methods take too long since they involve growing clones that are thought to be cold-tolerant in cold areas and then assessing their performance. The second main objective of this work was, therefore, to address this latter problem. The strategy employed was to investigate a biochemical response of cold-tolerant clones when subjected to low temperatures. In order to achieve this, a method for leaf protein gel electrophoresis was investigated and used to test whether or not polypeptides were produced by cold-tolerant clones in response to cold treatment.

CHAPTER 2

ESTABLISHMENT OF *In Vitro* PROTOCOLS FOR THE MICROPROPAGATION OF COLD-TOLERANT *Eucalyptus saligna* x *grandis* HYBRIDS

2.1 INTRODUCTORY REMARKS AND REVIEW OF LITERATURE

2.1.1 General aspects of *in vitro* cell and tissue culture

The prehistory of plant tissue culture began more than 225 years ago. The discovery of callus was by Henri-Louis Dunhamel Du Monceau (Mavituma, 1987). The cell theory which was expressed independently and almost simultaneously by Schleiden (1838) (Mavituma, 1987), with respect to plants, and that of Schwann (1839)(Mavituma, 1987) for both animals and plants, admitted implicitly that a cell is capable of autonomy and even that it is totipotent (Mavituma, 1987). A theoretical concept for cell culture was finally proposed in 1902 by the German botanist Harberlandt who predicted that one day 'one could successfully cultivate artificial embryos from vegetative cells'. The work of Harberlandt on single cells derived from palisade tissue of leaves, pith tissue of stems and the glandular and stamen hairs of *Tralescantia*, demonstrated that it was possible to support cells in a viable state for up to 20-27 days in a Knop's mineral solution containing sucrose, asparagine, and peptone. However, those cells did not divide although showing up to eleven-fold increase in original volume. The milestone for satisfying the major criteria of plant tissue culture, i.e. of potentially unlimited and undifferentiated growth, came when White (1943) achieved indefinite culture of tomato roots on a defined nutrient medium (Mantell *et al.*, 1985).

Plant tissue culture is a term commonly used to describe the methods of growing plant cells, tissues or organs *in vitro* isolated from the mother plant, on artificial medium. The small organs or pieces of tissue that are used are called explants (George, 1993/96). The techniques of tissue culture are, in effect, extensions of conventional propagation manipulations carried out on a miniature scale under aseptic conditions. For this reason, propagation *in vitro* is termed/called micropropagation.

Tissue culture has become an important technique for rapid multiplication (mass propagation) of selected varieties of plants. Application of tissue culture methods was initially confined to herbaceous plants (Murashige, 1977a; Murashige, 1978) and these methods are now extensively used commercially to mass produce ornamentals. Similar advances were not made in forest or other trees until the early 80s (Bonga, 1982; Bozini, 1980). However, this has changed in the last 15 years (Table 2.1) due to an increasing world population and rise in demand for tree products, especially wood and paper which, in turn, has increased the need to produce more timber through planting more forest with improved quality stock (Thorpe and Harry, 1990; Hamatt and Ridout, 1992).

2.1.2 Types of regeneration in tissue culture systems

There are two fundamental types of vegetative regeneration (i.e. routes of differentiation) in tissue culture systems, *viz.* a) organogenesis and b) embryogenesis. However, there are several *in* vitro culture systems that can be achieved via organogenesis. To list but a few, i) meristem-tip elongation, ii) axillary shoot production, and iii) adventitious shoot initiation. All the possible routes in tissues

culture are outlined in Figure 2.1. With regard to this discussion, only direct organogenesis and somatic embryogenesis will be discussed in detail as only these pertain to the present investigation.

Table 2.1: Examples of reported studies of work on tissue culture of forest species. Explant in column 2 reflects the starting material of the culture with the reported results in the successive column (3). The last column (4) gives the reference or authors of the particular studies.

Species	Explant	Result of studies	Reference
Acer rubrum 'Red Sunset'	2-3 node explants from *in vitro* cultures	Shoot proliferation. Shoots rooted	McClelland *et al.* (1990)
Albizia falcataria (L.) Fosb.	Cotyledon segments ex *in vitro* seedlings	Multiple shoots. Rooted	Kumar Sinha and Mallick (1993)
Bambusa vulgaris	Mature nodes	Budbreak	Nadgir *et al*(1984)
Betula pendula	Leaf sections ex *in vitro* and field plants	Multiple shoots. Rooted	Leege and Tripepi (1993)
Dalbergia sissoo	Mature nodes	Budbreak, plantlets	Datta *et al* (1983)
Dendocalamus strictus	Mature nodes	Budbreak, plantlets	Nadgir *et al* (1984)
Dipterocarpus intricatus	Seedling cotyledonary nodes	Axillary shoot multiplication. Rooted	Linington (1991)
Duboisia myoporoides	Leaves	Advent. buds, plantlets	Kukreja *et al* (1986)
Eucalyptus camaldulensis	Mature nodes	Budbreak, plantlets in soil and field	Gupta *et al* (1983)
	Mature leaves from *in vitro* shoots	Callus, adventitious buds, plantlets in soil	Muhalidharan and Mascarenhas (1987)
Fraxinus americana	Immature and mature seeds	Shoots and somatic embryos. Plantlets	Bates *et al.* (1992)
Fraxinus excelsior	Axillary shoots from young plants	Multiple shoots. Rooted	Hammatt and Ridout (1992)
Fraxinus excelsior	Axillary shoots from young plants	Shoot multiplication, but poor chlorophyll formation	Leforestier *et al.* (1991)
Fraxinus ornus L. (Manna ash)	Zygotic embryo	Germination and plantlet development	Arrillaga *et al.* (1992)
Garcinia mangostana	Segments of seed	Multiple shoots, 20%	Normah *et al* (1992)

L.		rooted plants	
Garcinia mangostana	Half seeds or slices	Single or multiple shoots dep. on medium	Teo (1992)
Poinclana regia	Anthers	Callus, embryogenesis	Gharyal and Maheshwari (1983)
Populus alba x *P. grandidentata*	Stem leaf and root seg. ex *in vitro* cultures	Shoots regenerated. Rooted plants	Son and Hall (1990a)
Populus alba x *P. grandidentata*	Root segments ex in vitro cultures	Multiple shoots. Rooted	Son and Hall (1990b)
Populus x *canadensis* [5 clones]	Lateral buds	Shoot /growth proliferation	Antonetti and Pinon (1993)
Populus deltoides	Segements of cultured adv. shoots	Stable shoot proliferation	Coleman and Ernst (1990)
Populus tremula clone KWS 3 17	Roots	Numerous adventitious shoots formed	Nadel *et al.* (1992)
Quercus petraea	Immature zyg. embryos	Some somatic embryos	Chalupa (1990)
Quercus suber L. (Cork oak)	Apical/nodal seg. ex seedlings or adult trees	Shoot multiplication and rooting	Manzanera and Pardos (1990)
Quercus robur	Juvenile and rejuvenated shoot tips	Multiple shoots, rooted	Vermeer *et al.* (1991)
Quercus rubra L. (Red oak)	Male catkins	Callus degenerated after 5 months. No embryoids	Gingas (1991)
Quercus rubra	Juvenile and rejuvenated shoots	Shoot growth during at least six subcultures	Rancillac *et al.* (1991)
Sesbania grandifolia	Hypocotyl, cotyledon	Callus, adventitious buds, plantlets	Khattar and Mohan Ram (1983)
Sesbania aculeata (Poir) Prickly sesban	Seedling hypocotyl sections	Multiple shoots. Shoots rooted	Bansal and Pandey (1993)

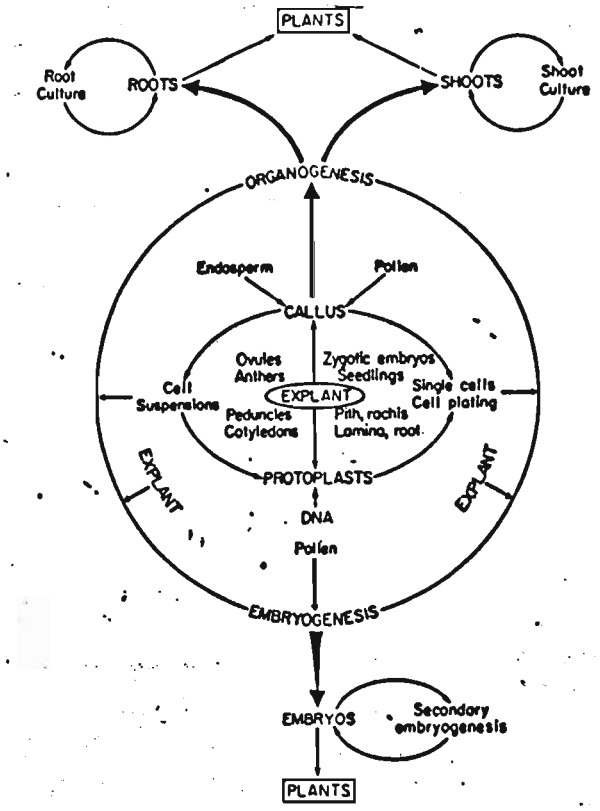


Figure 2.1: Cultural manipulations now possible through tissue culture technique. (Adapted from Mantell *et al.*, 1985 by Blakeway, 1992)

a) Organogenesis

Morphogenetic meristems can theoretically occur in either of two distinct ways: 1) from the differentiated cells of a newly transferred piece of whole-plant tissue, without the proliferation of undifferentiated tissue, or 2) from the unspecialised, unorganised and dedifferentiated cells of callus tissues or suspension cultures. Hicks (1980) refers to these two methods of morphogenesis as direct and indirect organogenesis, respectively. Direct shoot organogenesis (or shoot morphogenesis) involves the development of axillary or adventitious buds following the inhibition of apical dominance. Alternatively, shoot meristem may form indirectly within callus [defined by Skoog and Miller (1957) as a mass of tissue with a low level of

organisation] which is still attached to, or within, the tissues of the primary explant, or may be produced simultaneously from such callus and from superficial cells of the explant (Bigot *et al.*, 1977; Shimada and Yamada, 1979; Evans *et al.*, 1984; Bhaskaran and Smith, 1990).

With regard to the present investigation, the most important culture system that is based on the (direct) organogenesis route is axillary bud proliferation. In this type of regeneration, the apical meristem and lateral growing points on the explant and the nodes below the apical meristem are stimulated to grow. Success varies with the explant used and depends on the application of appropriate hormones. The explant may be all or part of an apical or lateral growing point on a stem or it may be a stem section of several nodes (Murashige, 1974; Murashige, 1977b; Zilis *et al.*, 1979; Henny *et al.*, 1981). Growth from these axillary shoots provides a rapid multiplication system in which the number of potential plants is increased exponentially by repeated culturing. Production of axillary shoots may be accompanied by proliferation of adventitious buds from callus on the base of a propagule (Abbott *et al.*, 1976). In such cases, higher rates of multiplication can occur since the increase is not dependent on the number of nodes.

Shoot tips from trees, or other woody perennials, can be difficult to decontaminate. Because of this, Standardi and Catalano (1985) preferred to initiate shoot cultures of *Actinidia chinensis* from meristem tips which could be sterilised easily. De Fossard *et al* (1977) could initiate cultures of *Eucalyptus ficifolia* from shoots from 36 year-old trees, but forest-gathered material was very difficult to decontaminate unless covered

and protected for some period before excision. Furthermore, shoot tips of woody plants are more liable than those of herbaceous species to release undesirable phenolic substances when first placed onto a growth medium.

Regeneration of plants is the final step in the process of clonal propagation via tissue culture systems, and it is the critical step to determine whether or not the system is successful (Huang *et al.*, 1993). Since the pioneer regeneration work reported in *Populus tremuloides* and *Pinus palustris* via organogenesis in the early to mid 1970s, micropropagation techniques have been developed and refined for many woody species, including both gymnosperms and angiosperms (Table 2.1). Of tree species, gymnosperms such as mature pines, spruces and firs and some angiosperms such as oaks, maples, beeches, and walnuts continue to be difficult to regenerate *in vitro* (Haissig, 1989). The genus *Eucalyptus* itself has been investigated by many workers and a review of this work is presented in section 2.1.6.a. Success in tree regeneration greatly depends on the choice of tissue source, age of the tree, and species and genotype chosen (Huang *et al.*, 1993). Finally, trees regenerated from a propagation system must perform satisfactorily in the field tests to prove their practical value the tree improvement programs.

b) Embryogenesis

Somatic embryogenesis can be defined as the production of embryogenetic-like structures from somatic or asexual cells (Steward *et al.*, 1958; Haccius, 1978; Schwendiman *et al.*, 1990; Tulecke, 1987; Warren, 1991; Raemakers *et al.*, 1995). A somatic embryo is an independent bipolar structure, not physically attached to the

tissue of origin and develops and germinates to form a plant in a manner analogous to germination of zygotic embryos in seeds (Ammirato, 1987; Emons, 1994) (Figure 2.2).

Direct embryogenesis utilises, as explants, tissues that are immediately able to produce somatic embryos. Examples of these are nucellar tissue of polyembryonic citrus cultivars (Button and Kochba, 1979; Huang *et al.*, 1990), grape (*Vitis*) ovules (Mullins and Srinivasan, 1976), and immature cocoa (*Theobroma*) embryos (Pence *et al.*, 1979), although this type of embryogenesis may also involve some callus formation. These explants are said to have pre-embryonically determined callus (PEDC) (Sharp *et al.*, 1980; Evans *et al.*, 1981; Sharp and Evans, 1982). Indirect embryogenesis utilises an intermediate stage callus, or a suspension culture during which a change in potentiality of the cells is induced. Such somatic embryos are described as arising from induced embryonically determined cells (IEDC) (Sharp *et al.*, 1980; George, 1996). Some authors favour the idea that the two processes are actually the same and somatic embryos arise from the proliferation of special cells in the original explant (Street, 1977). In either case, embryo-producing (embryogenic) callus, once developed, continues to produce embryos (Krul *et al.*, 1977; Krul *et al.*, 1980).

Somatic embryos, therefore, arise from single cells located from within clusters of meristematic cells either in the callus or in the suspension (Steward *et al.*, 1970; Williams and Maheswaran, 1986; Tulecke, 1987; Warren, 1991; Emons, 1994). Somatic embryo formation was first induced in relatively few plant species (Tisserat

et al., 1979), but its incidence is steadily being increased as controlling factors become better understood (Williams and Maheswaran, 1986).

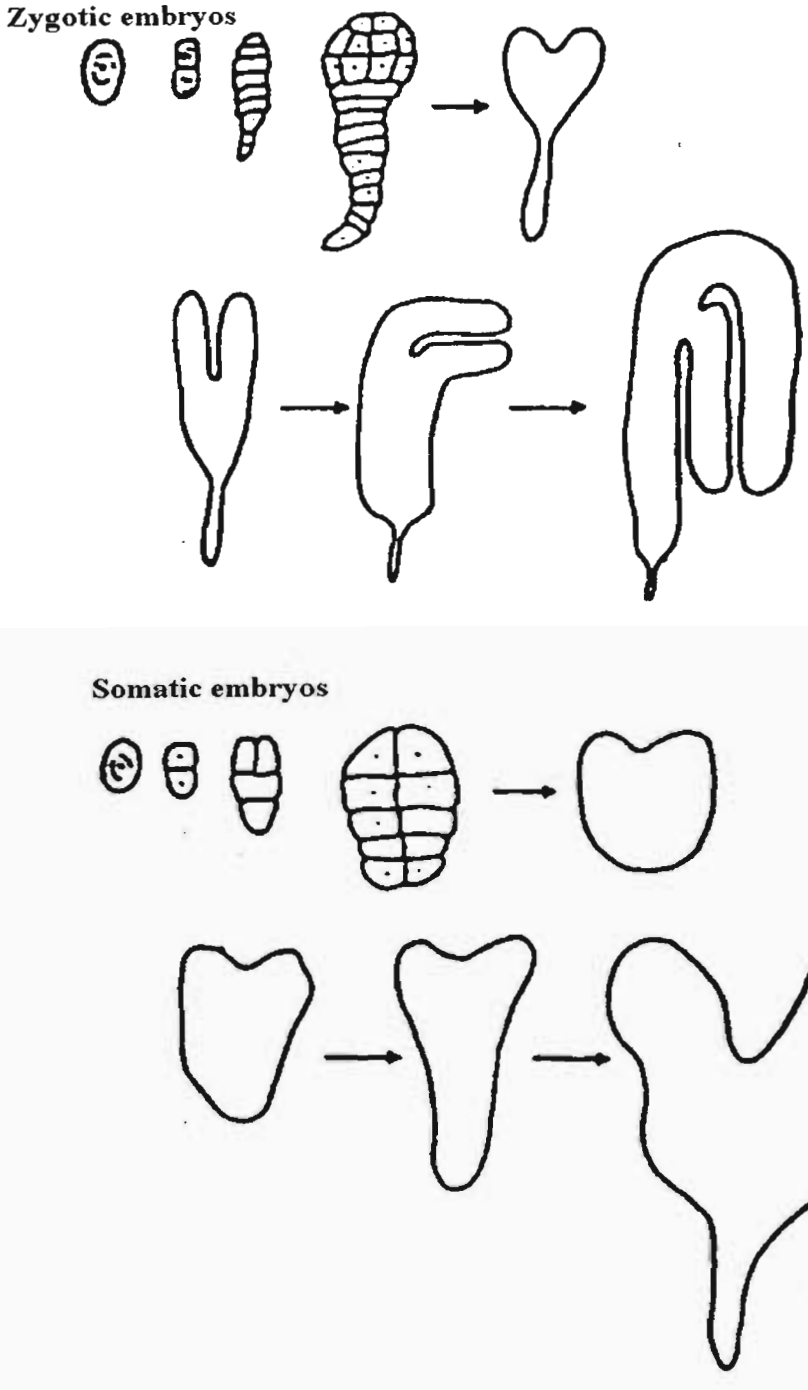


Figure 2.2: Diagrammatic representation of the stages in zygotic and somatic embryogenesis in carrot. (reproduced from Lindsey and Jones, 1990).

In woody species, investigations of somatic embryogenesis have led to rapid progress during the last few years (Table 2.1).

2.1.3 Some of the factors affecting tissue culture success

2.1.3.1 The explant

An explant is a piece of tissue or organ which is removed from the plant for purposes of culture. Success in culturing the explant is influenced by a number of factors inherent to the explant, including the genotype of the explant, *viz.* its size, its physiological age, and the tissue or organ source of the explant (Ammirato, 1983a; George *et al.*, 1983; Cohen, 1986; Tulecke, 1987; McGee *et al.*, 1989).

a) Explant size

As a general rule, very small explants have low survival rate in culture. This is true for both tissues and callus (Conger, 1981). In studies with carnation, shoot apices measuring 0.09 mm were incapable of morphogenesis, but when the explant was as large as 0.5 mm, shoot production was again reduced, possibly due to the presence of subapical tissue (Gukasyan *et al.*, 1977). Presumably, if the explant measured between 0.1 and 0.4 mm then morphogenesis was succesfully achieved. In a study with *Chrysanthemum*, shoot tips between 0.2 mm and 0.5 mm and shoot meristems between 0.1 mm and 0.2 mm produced only a single shoot, whereas larger explants, 0.5 mm to 1.5 mm in length, produced multiple shoots (Wang and Ma, 1978). In most studies, therefore, the researcher needs to establish the optimal size of explant to be used.

b) Source of explant

Within any plant, tissues differ in their degree of determination and thus in their ability to undergo morphogenesis (Conger, 1981; Ammirato, 1983a; Tulecke, 1987). Meristematic tissues or organs should be selected in preference to other tissue sources because of their clonal properties, culture survival, growth rates and totipotentiality *in vitro* (Quak, 1977; Tisserat, 1987). Tissues that exhibit morphogenic competence in one taxon may be equally competent in other taxon. Bulbs or corms (Jha *et al.*, 1991), young leaves (Stamp and Henshaw, 1987a; Szabados *et al.*, 1987: Raemakers *et al.*, 1993), inflorescence stems (Jha *et al.*, 1991; Beruto and Debergh, 1992), and embryos (Stamp and Henshaw, 1982; Stamp and Henshaw, 1987b; Raemakers *et al.*, 1993) can be used as sources of explant as reported in many studies. Bonga (1982) reported that early trials to culture Douglas-fir could not succeed due to difficulties in culturing tissues from adult specimens.

Somatic embryogenesis and plant regeneration has been attained from leaves and inflorescence stems (Stolarz *et al.*, 1991; Baker and Wetzstein, 1992; Beruto and Debergh, 1992; Sorvari *et al.*, 1993; Lainé and David, 1994); from cotyledons (Tulecke and McGrahanahan, 1985; Jha *et al.*, 1992;; Ozias-Akins *et al.*, 1992; Saravitz *et al.*, 1993;) and from seeds and embryos (Vasil and Vasil, 1982; Guimaraês *et al.*, 1988; Jain *et al.*, 1989; Gui *at al.*, 1991; Tautorus and Schel, 1991; Torné *et al.*, 1992).

Most of the published work on plant regeneration from *Eucalyptus grandis* (section 2.1.6) concerns the use of seedling material as explants (Watt *et al.*, 1991; Warrag *et*

al., 1991). It has long been known that studies on morphogenesis in higher plants is further complicated by different nutritional and growth regulator requirements for the process by different types of explants Buys *et al* (1966). For example, carnation hypocotyl and shoot-tip explants were shown by Petru and Landa (1974) to differ in media requirements for growth and morphogenesis.

2.1.3.2 Media

One of the most important factors governing the growth and morphogenesis of plant tissues in culture is the composition of the growth medium. Several plant tissue and cell culture media are in common use including formulations derived by White (1963), Murashige and Skoog, (1962) and Gamborg *et al.* (B5 medium) (1968). The Murashige and Skoog formulation is often used when morphogenesis is required. When rapid cell proliferation is desirable, B5 medium, particularly in combination with 2,4-dichlorophenoxyacetic acid (2,4-D) is used. In general, plant tissue culture media consist of mineral salts, a carbon and energy source, vitamins and growth factors. Other organic compounds may be included (Conger, 1981).

a) mineral salts

The salt composition of several media has been reviewed by Huang and Murashige (1977). The Murashige and Skoog (MS) formulation, probably the most popular medium for horticultural studies, is high in nitrates, potassium, and ammonia (Conger, 1981). Iron is an essential medium component for growth and morphogenesis of many species (Nitsch, 1969). That author also demonstrated that *Nicotiana tabacum* plantlets would not form from microspores unless iron was added

to the medium. However, iron ethylene diaminotetracetic acid (EDTA) as well as other EDTA chelates are not entirely stable in liquid cultures, particularly after autoclaving, and may combine with other compounds to form a precipitate after a few days (Stoltz, 1979). High levels of nitrogen were found to make the formulation suitable for use in somatic embryogenesis protocols. Nitrogen should be in the form of ammonium nitrate in MS medium as it is highly required by developing embryos (Kirby *et al.*, 1987).

b) carbon and energy source

The preferred carbon source for most *in vitro* culture systems is sucrose (George, 1993/96). The quantity and the form of the carbohydrate used as an energy source is one of the major factors that contributes to the success of explant development (Ammirato, 1983a; Thompson and Thorpe, 1987; Levi and Sink, 1990; Romano *et al.*, 1995). For example, Tran Thahn Van (1977) has investigated the effects of various sugars on flower formation from thin layer epidermal sections of tobacco. At 0.08 M, the best response was obtained with either saccharose plus glucose or saccharose plus fructose. At 0.17 M, glucose alone and saccharose plus glucose were equal in ability to induce flower induction. A similar approach was recently followed by Cabasson *et al.* (1995) who conducted studies aimed at controlling somatic embryogenesis on solid medium using various carbon sources that have been found to promote somatic embryo development in different species. These included sorbitol and maltose (carrot: Wetherell, 1984; Kinnersly and Henderson, 1987), maltose (alfalfa : Strickland *et al.*, 1987), sorbitol and mannitol (*Viginia aconitifolia* : Kumar *et al.*,

1988), mannitol (celery : Nadel *et al.*, 1989), fructose and galactose (muskmelon : Oridate and Yazawa, 1990), and glycerol (*Cichorium* : Robatche-Claive *et al.*, 1992).

c) Vitamins

Linsmaier and Skoog (1965) demonstrated that for *Nicotiana tabaccum*, most vitamins are not essential for callus growth. Pyrodoxine, nicotinic acid, and biotin could be deleted from the medium without the loss of growth (Conger, 1981). That author also reported the inhibition of growth by riboflavin whereas folic acid and p-amino-benzoic acid (pABA) increased growth, but were not essential. Thiamine (0.1 to 0.5 mgl^{-1}) is almost always essential for callus growth (Conger, 1981; Hartmann and Kester, 1983) and nicotinic acid (0.5 mgl^{-1}) and pyrodoxine (0.5 mgl^{-1}), required for some plant tissues, are usually added to the medium for callus growth. Inositol at 100 mgl^{-1} is beneficial in many cultures and also is added routinely (George, 1993/96). All of these substances are soluble in water and should be prepared as stock solutions, ready for dilution, at 100x the final concentration. These stock solutions should be stored in a refrigerator (Hartmann and Kester, 1983).

d) Growth regulators

Some chemicals occurring naturally within plant tissues (i.e. endogenously), have a regulatory, rather than a nutritional role in growth and development. Such compounds, which are generally active at low concentrations, are known as plant growth substances (PGS) (or hormones). Synthetic chemicals with similar physiological activities to PGS, or compounds having an ability to modify plant growth by some other means, are usually termed plant growth regulators (PGR)

(George, 1993/96) and are applied exogenously. There are several recognised classes of plant growth substance: i) auxins ii) cytokinins iii) gibberellins iv) ethylene and v) abscisic acid.

Auxins and cytokinins are by far the most important for regulating growth and morphogenesis in plant tissues and organ cultures (George, 1993/96; Murthy and Saxena, 1994). Combinations of growth regulators have been reported several investigations including those in woody species (Watt *et al.*, 1991; Tuomi and Rosenqvist, 1995; Dimasi-Theriou and Economou, 1995). Early studies by Skoog and Miller (1957) which indicated that auxin to cytokinin ratios determined the type and extent of organogenesis, have greatly influenced subsequent investigations. The commonly used auxins are 3-indole acetic acid (IAA), 3- indolebutyric acid (IBA), 2,4-dichlorophenoxyacetic acid (2,4-D), 2,4,5- trichlorophenoxyacetic acid (2,4,5-T), 4-chlorophenoxyacetic acid (CPA), and 4-amino-3,5,6-trichloropicolinic acid (pichlororam or TCP). Based on stem curvature assays, as compared to IAA activity, 2,4-D is eight to twelve; 2,4,5-T and CPA are four and two times stronger, respectively (Thimann, 1951). Synthetically prepared IAA is used as an auxin in plant tissue culture media, but it tends to be denatured in culture media. However, in some plants, callus induced by IAA (together with cytokinins) frequently gives rise to shoots or embryos as its effective concentration becomes diminished (George, 1993). Indoleacetic acid has also been used with other regulants to induce direct morphogenesis and for meristem and shoot cultures e.g. *Bougainvillea*, (Chaturvedi *et al.*, 1978; Sharma *et al.*, 1981) *Citrullus*, (Barnes, 1979) and *Sinningia* (Haramaki, 1971; Grunewaldt, 1977).

Cytokinins comprise a separate class of growth substances and growth regulators. They produce only minor effects when applied to intact plants, but have been noted to stimulate protein synthesis (George, 1993/96). Frequently utilized cytokinins are 6-benzyladenine (also 6-benzylamino purine, BA, BAP), 6-γ-γ-dimethylallyaminopurine (also isopentenyl-adenine, 2iP, IPA), 6,furfuryl-aminopurine (kinetin) (KIN), and 6-(4-hydroxy-3-methyl-trans-2-butenylamino) purine (zeatin). Cytokinins have been found to be more important in somatic embryogenesis and embryo maturation (Fujimura and Komamine, 1980; Vasil and Vasil, 1981b; Ammirato, 1983b; Carman *et al.*, 1988). Trindale *et al* (1990) reported in their study on the role of cytokinin and auxin in rapid multiplication of shoots of *Eucalyptus globulus*. Those authors found that exogenous cytokinin was a major limiting factor and controlling factor for shoot multiplication grown in culture. Benzylaminopurine was effective than kinetin in promoting shoot formation. Auxin (IBA) was also more important and could compensate for low levels of cytokinin.

Plant tissue cultures can generally be induced to grow and differentiate without gibberellins, although gibberellic acid (GA_3) may become an essential ingredient of media for culturing cells at low densities. High concentrations of GA_3 (e.g. greater than *ca.* 1-8 mg/l) induce the growth of undifferentiated callus cells (Schroeder and Spector, 1957; Murashige, 1964; Mehra and Mehra, 1972; Altman and Goren, 1974; Beasley, 1977, Gautam *et al.*, 1983), and can promote the growth of callus in combination with auxin and low levels of cytokinin (Engelke *et al.*, 1973).

The presence of very low concentrations 0.1 v.p.m [parts per million per volume] of ethylene gas in the atmosphere has long been known to cause plants to grow abnormally (Fidler, 1960; Heck and Pires, 1962a,b; Chadwick and Burg, 1967). The gas is most obviously involved in fruit ripening, senescence, and the abscission of leaves, but also has many other functions (Hayes, 1981). Ethylene does not seem to stimulate callus growth of some plants *in vitro* e.g., on a hormone-free medium, *Ginkgo biloba* embryos germinated within tubes scaled with cotton plugs, but gave rise to callus if the tubes were covered with Parafilm (Webb *et al.*, 1986). Thorpe *et al.* (1981) and Huxter *et al.* (1981) have suggested that ethylene inhibits shoot formation during the first five days of culture initiation period, but thereafter it speeds up the formation of primordia.

Abscisic acid (ABA) is another naturally-occurring growth substance. It is derived by plants from mevalonic acid (MVA), or from the breakdown of carotenoid pigments (Neill and Horgan, 1984). It is usually inhibitory to callus growth (von Arnold *et al.*, 1994). Sankhla and Sankhla (1968) reported that 1 mgl^{-1} was markedly inhibitory to *Ipomea* callus, but generally concentrations between 5 and 50 mgl^{-1} appear to be necessary to cause a 50% inhibition of cell growth (Li *et al.*, 1970; Gamborg and LaRue, 1971, Taylor and Burden, 1972). Several independent instances have been cited where ABA has been capable of stimulating callus growth, e.g. on hypocotyl explants of *Cryptomaeria* (a gymnosperm tree), 1 mg/l ABA in the culture medium had the same effects as 10 mgl^{-1} of the cytokinin BAP in stimulating internal callus or adventitious bud formation (Isikawa, 1974). There are also reports of low concentrations of ABA stimulating somatic embryo initiation or embryo growth. For

example, embryogenesis was stimulated in growth regulator-habituated *Citrus sinensis* (Shamouti orange) callus by 0.01 to 1 mg/l ABA (Kochba *et al.*, 1978; Spiegel-Roy and Kochba, 1980). Abscisic acid, which is recognised as playing an important role in seed development (Le Page-Degivry and Bulard, 1988), was identified as an important medium component for maturation of somatic embryos in several conifer species (Hakman and Arnold, 1985, 1988; Durzan and Gupta, 1987; Boulay *et* al., 1988, Dunstan *et* al., 1988, Veisseire *et al.*, 1994). Somatic embryos with well-developed cotyledons, which in their morphology appear to resemble zygotic embryos, have been obtained on an maturation medium supplemented with ABA (Lelu *et al.*, 1994a).

2.1.3.3 Subculture

There is increasing evidence that extended subculturing of callus or cell suspension cultures can lead to a reduction in morphogenic potential (e.g. Vasil *et al.*, 1964; Chen and Galston, 1967; Hill, 1967; Reinert and Backs, 1968; Pearson, 1979; George, 1996). Experiments with *Convolvulus* callus showed that after three or more subcultures, some lines lost morphogenic ability (Hill, 1967). Reinert and Backs (1968) observed a gradual loss of morphogenic potential in long-term carrot cultures. However, restoration of regenerative ability in carrot cultures with the addition of kinetin was reported by Wochok and Wetherell (1972). In contrast, some tissue and cell cultures retain morphogenic potential over extended periods of subculturing. *Chrysanthemum*, propagated by callus containing meristematic areas, retained morphogenic potential over a three and a half year period (Earle, 1974). Loss of morphogenic potential might be result of reduced endogenous levels of growth

regulators. This view is supported by studies with *Isatis tinctoria* and carrot cultures (Dancwardt-Lilliestrom, 1957; Wochock and Wetherell, 1972).

Continuous subculture often increases the frequency of aberrant plants arising from the cultures (Zimmer and Pieper, 1976; Ben-Jaacov and Langhams, 1972; Skirvin and Janick, 1976; Nishi *et al.*, 1968; Malnassy and Ellison, 1970). Often the variability is associated with callus formation (Skirvin and Janick, 1976; Gukasyan *et al.*, 1977) and somaclonal variation (section 2.1.5.b). The range of variability is wide, phenotypic changes observed in plants derived from callus of the scented geranium included changes in leaf morphology, loss of pubescence, dwarfing, changes in essential oil constituents and flower morphology. However, while variability may be undesirable from a propagator's point of view, it serves as a pool from which desirable traits may be selected (Matern *et al.*, 1978) (section 2.1.5.b).

2.1.3.4 Light

The culture of plant cells on medium containing sucrose should bypass the need for photosynthesis and, in fact, cells of numerous species are routinely cultured in the dark. However, several studies have demonstrated that light plays an important role in inducing organogenesis. In the Iridaceae, callus cultured in the dark was found to produce shoots only when transferred to light (Simonsen and Hilderbrandt, 1971; Bajaj and Pierik, 1974; Hussey, 1976). Experiments with *Nicotiana tabacum* indicated that light was a critical factor in the initiation of shoots (Murashige and Nakano, 1968) and that it also influenced root growth (Murashige and Nakano, 1968). Production of somatic embryos has been achieved under a wide range of light

regimes. The quality, quantity and duration and the light/dark regimes have an influential role on somatic embryogenesis (Sondahl and Sharp, 1979; Tulecke, 1987). Watt *et al.* (1991) reported that in *Eucalyptus*, dark conditions favoured the induction of embryogenesis whereas germination occurred under a light/dark regime.

Early studies with *Bryophyllum tubiflorum* and *B. daigremontianum* indicated that photoperiod affected bud formation (Heide, 1965; Sironval, 1965). The effective photoperiod for morphogenesis varies between taxa. A 12h photoperiod was optimal for shoot production from *Helianthus* tuber sections (Gautheret, 1969), and 16h of light was most favourable for induction of flowers from thin layer epidermal sections.

Since wavelength-specific morphogenic responses are common in the plant kingdom, it is therefore not surprising that they should be observed *in vitro*. Wavelength ranging between 400-730 nm has been observed to have different roles in on bud formation (Kato, 1978). Bud induction of pine cotyledons was maximised at 660 nm, while purple and near-UV light had no effect (Kadkade *et al.*, 1978). Murashige (1974) has reported that optimum light intensity for plant tissues in culture may differ from the requirements of plants themselves.

2.1.3.5 Temperature

Early studies on the growth of plant cells in culture indicated that the optimum range was between 26 and 28 °C, but the species requirements differed considerably (Carew and Staba, 1965; Puchan and Martin, 1971; George, 1993/96). This is not surprising as the effects of temperature on plants are largely mediated by their effects on

chemical reactions (Went, 1953). Studies on the growth of suspension cultures of *Impomea* at temperatures from 15 to 34 °C indicated that maximum growth of the cultures occurred between 25 and 32 °C. Growth of *Narcissus* explants (Seabrook and Cumming, 1978) and induction of roots on *Rhododendron* explants (Pierik and Steegmans, 1975) was best at 25 °C. Temperature around 20 °C seems to be suitable for root induction of conifers (Campbell and Durzan, 1976; Chalupa, 1977; Arnold, 1982; Chalupa, 1983). However, a low temperature may trigger specific morphogenetic events. For example, a temperature treatment of 17 °C was necessary for the *in vitro* induction of gametangia in *Physcomitrella patens* and 7 °C in *Funaria hygrometrica*, but no gametangia were formed in either at 24 °C. Bonga (1977) found that pine callus cultures occasionally cease growth and become dormant if continuously exposed to optimal constant temperatures.

Although there are collected papers documenting the environmental control of micropropagation (Kozai *et al.*, 1992), little has been documented on the effects of temperature on somatic embryogenesis.

2.1.4 Hardening-off of regenerated plantlets

This stage involves the transfer of the plantlet from the aseptic culture environment to free-living environment of the greenhouse and ultimately to the final location. Several environmental conditions are essential in the initial period after transplanting (Smith, 1981). Most plantlets derived *in vitro* by organogenesis or somatic embryogenesis, survive and can be grown to maturity after transfer to soil (Vasil and Vasil, 1984; Ziv, 1986). However, never having been exposed to normal

environmental conditions, plants produced by tissue culture are accustomed to high humidity levels and aseptic conditions, so they need to be hardened-off with gradual exposure to the environment (Ziv, 1986). Acclimatisation can be improved by using fog equipment or by increasing CO_2 levels (Maene and Debergh, 1987). In addition, conditions during propagation and rooting phases may also enhance the performance after planting. Reduction of humidity in containers by using closures that allow water vapour escape or bottom cooling (Maene and Debergh, 1987) may result in plants with improved water retention capacity.

For *Eucalyptus* spp., Poissonnier *et al.* (1984) have clearly demonstrated that success in acclimatizing *in vitro* plantlets to greenhouse conditions greatly depends on the quality of the *in vitro* plant. In woody species, a humid confinement is often preferable to mist (Boulay, 1984), although *Eucalyptus* spp. acclimatize well under mist conditions (Blakeway, per. comm.).

2.1.5 Potential applications of tissue culture

Tissue culture and its probable future developments, have the potential to accelerate the genetic improvement of forest yield. This could be possible if this technology could be integrated with conventional tree breeding and selection programs. Some of the potential applications of *in vitro* culture systems have been discussed in detail by various authors and include large scale micropropagation (Bonga and Durzan, 1982; Nashar, 1989), crop improvement by somaclonal variation (Larkin and Scowcroft, 1981), germplasm storage (Kartha, 1982; Withers, 1983; Lindsey and Jones, 1990),

production of haploid plants (Lindsey and Jones, 1990; Vasil, 1990) and virus and pathogen elimination (Ingram and Hegelson, 1980).

a) Large-scale micropropagation

The main advantage of micropropagation over traditional propagation methods is that large number of plants can be quickly produced in a small area all year round (Nashar, 1989). Hamatt (1992) reported that as a tree ages and becomes mature, its growth rate usually slows and it becomes progressively more difficult to propagate from conventional vegetative propagation macrocuttings. The potential yields of micropropagation are also much higher than those of macropropagation.

One of the most reliable types of micropropagation is achieved through axillary shoot production, which was discussed in previous sections (section 2.1.2). This process is based upon the principle that a bud taken from a given specimen, and cultured on a nutrient medium containing growth regulators, develops into a shoot and its axillary buds grow out to form side branches. These side branches can be rooted or transferred to fresh multiplication medium in which axillary buds can grow again (Hamatt, 1992). This process can be carried out indefinitely to produce limitless number of shoots. Somatic embryogenesis offers an alternative and also efficient system for mass clonal propagation as (theoretically) every cell in the explant or callus has the potential to regenerate into an embryo (Ammirato, 1989; Attree and Fowke, 1993; Rout *et* al., 1995). Several large-scale clonal propagation programmes are under way in which elite specimens of certain crops are being micropropagated (Lindsey and Jones, 1990), and information is now slowly being gathered on the

micropropagation of an increasing range of fruit and timber trees (Hamatt, 1992). For forest trees, most success in large-scale propagation has been reported in conifers, such as spruces (*Picea* spp.; Baxter *et al.*, 1989) and colonising broad-leaved species such as birches (*Betula* spp.; Valoba and James, 1990). For many important hardwood genera, such as tropical Dipterocarps (Linington, 1991), maples (*Acer* spp.; Preece *et al.*, 1991a, b), ashes (*Fraxinus* spp.; Hamatt and Ridout, 1992) and oaks (*Quercus* spp.; Manzanera and Pardos, 1990), little information has been published. Large-scale micropropagation of *Eucalyptus* may contribute in improving the productivity of plantations so that the same amount of wood is produced in a smaller area (Turnbull, 1991; Hope, 1993). The use of direct organogenesis would be the method of choice for mass propagation, since somaclonal variation would be minimised (Hope, 1993). Another possibility for large-scale micropropagation of eucalypts is the production of artificial seeds by the encapsulation of somatic embryos (Redenbaugh *et al.*, 1987), which would reduce the cost of seeds dramatically (Gupta *et al.*, 1993).

b) Crop improvement by somaclonal variation

Somaclonal variation is a term coined by Larkin and Scowcroft (1981) to cover all types of variations which occur in plantlets regenerated from cultured cells or tissues (Lindsey and Jones, 1990; Smith and Drew, 1990; Huang *et al.*, 1993). Hamatt (1992) refers to somaclonal variation as the process of adventitious regeneration that often results in heritable mutations, of which only a small proportion may be useful. Using this system, successful selection of mutants with various desired characteristics has been reported in some plant species. These include increased herbicide tolerance

in hybrid poplar and other species (Chaleff, 1986; Michler and Haissig, 1988), disease resistance in *Larix* and other species (Sacristan, 1986; Diner, 1991), as well as multi-gene agronomic characters. In trees, Lester and Berbee (1977) first detected somaclonal variation as variation in height, branching and leaf traits amongst regenerant poplar plants, while Ostry and Skilling (1988) later described poplar somaclones more resistant to the leaf spot fungus *Septoria musiva.* Hammerschlag (1988) produced peach plantlets which were more resistant than the parental tree to the bacterial pathogen *Xanthomonas* campestris var. pruni, while Donovan (1991) generated apple trees resistant to fireblight caused by *Erwinia* amylovora in the susceptible scion variety 'Greensleeves'.

Several mechanisms may be responsible from induction of somaclonal variation. These include the gross karyotypic changes which accompany *in vitro* culture via calli, cryptic chromosomal rearrangements, somatic crossing-over with sister chromatid exchange, transposable elements, gene amplification or diminution or perhaps various combinations of these processes (Mantel *et al.*, 1985; Huang *et al.*, 1993). Different types of cryptic chromosomal arrangements are well described aspects of meiotic behaviour of chromosomes in plants regenerated from cell cultures. These include reciprocal locations, deletions, inversions and non-homologous translocations and acentric and centric fragment formations (Flashman, 1982; George, 1996). Such rearrangements probably cause losses of genetic material or at the very least realignment and transportation of chromosomal material.

c) Storage of germplasm

There is a need for culture storage, by both conventional and novel techniques, in plant breeding and in the *in vitro* production of secondary compounds (Withers, 1988). Storage of genetic materials has become increasingly important for developing new cultivars as well as preserving heirloom varieties and rare or endangered species (Reed, 1992). Progress in several different areas like the generation of variation, the selection transfer of genes, the identification of desirable traits and the conversion of *in vitro* cultures to plants, contributes to the increased efficiency. However, these efforts are wasted if the production of *in vitro* procedures are prone to variation or, worse, accidental loss (Kartha, 1981; Dodds and Roberts, 1985; Krogstrup *et al*, 1992). Thus, germplasm preservation must be a component of any *in vitro* programme where genetic identity and integrity are important (Chen and Kartha, 1987; Ahuja, 1989; Lindsey and Jones, 1990).

Various methods of *in vitro* storage of germplasm are thus of great practical interest for longer-term storage of germplasm, and good progress has been made in this area. There are two main approaches to *in vitro* germplasm storage, namely; a) slow growth technique and b) cryopreservation. Cultures can be induced into a state of slow growth by various means, including culture at a reduced temperature or in the presence of growth regulators (Staritsky *et al.*,1986; Withers, 1987a, b). Cultures are maintained at 4-8 °C, usually with reduced light (e.g. an 8 hour day) (Lindsey and Jones, 1989). Reduced lightening may be advantageous, but there has been little systematic investigation on this aspect. In difficult cases, it can help to avoid a

storage temperature that is too low, to combine two stresses e.g. temperature and osmotic, or to warm cultures intermittently (Staritsky *et al.*, 1986).

There is only one approach that completely arrests tissue growth, and that is the reduction of temperature of biological material to that approaching the temperature of liquid nitrogen. The term 'cryopreservation' is used exclusively to define this kind of approach. At this temperature (-196 °C), almost all metabolic activities are standstill and can be preserved in that state almost infinitely (Yamada *et al.*, 1991; George, 1993/96). A typical cryopreservation procedure consists of the following stages: pregrowth, cryoprotection, freezing, storage, thawing and recovery. the exact treatments given at each stage will vary with culture system (Kartha, 1985; Sakai, 1986; Withers, 1985, 1987a, b; Reuff *et* al., 1988; Lindsey and Jones, 1990). Except for some orthodox seeds, dormant buds and pollen, higher plant structures cannot generally survive the transition too and from the storage temperature without protection.

The advantages of *in vitro* conservation include the fact that these methods are extremely convenient in terms of labour costs and space utilisation since they require little or no maintenance at all (Wilkins *et al.*, 1992, Hope, 1994). This is important in forest and other tree species which would take up vast areas if storage was done in the form of field collections. Krogstrup *et al.* (1992) also argues that it becomes a necessity to have an alternative storage method available for species and hybrids where seeds are not set or readily available for storage.

d) The production of haploid plants

Haploid plant production using pollen and anther culture techniques is an obvious benefit derived from tissue culture application and has been dealt with at great length in reviews by Chu (1982) and Collins and Genovesi (1982). In the breeding context, haploids *per se* are not useful as sources of homozygous lines (Liu, 1984). However, by tissue culture, haploids can be produced in a matter of months in large amounts and their chromosomes complements doubled to generate the homozygous lines necessary for breeding (Mantell *et al.*, 1985). The number of plant species from which anther culture has resulted in haploid plants is relatively few (George, 1996). Species in which haploid plants can be regenerated reliably and at high frequencies include *Datura*, *Hyoscymus*, and *Solanum* (George, 1996). Guha and Maheshwari (1964, 1967) managed to regenerate haploid plants from pollen of *Datura innoxia* by culturing intact anthers.

e) Production of pathogen-free plants

This is a specialised area of micropropagation which is based on general aseptic culture procedures and meristem tip and shoot tip culture techniques (Ingram, 1980). Diseases of crop plants caused by pathogens such as viruses, viroids, fungi, nematodes, bacteria, and mycoplasma are transmitted by vegetative propagation procedures. Therefore, there is frequently an essential need to eradicate pathogens from certain elite lines of plant material (Wang and Charles, 1991).

In meristem culture, the more differentiated vascular tissues occur away from the meristem (towards the older tissues of the stem), the vascular elements of the leaf

primordia are still incipient, and they have not yet made contact with the main strand of the vascular system of the stem. Therefore, virus particles which may be present in the vascular system can reach the meristematic region of the apex only through cell-to-cell movement. Meristem tip culture has been used for the elimination of viruses for several plant species (Roca, 1986, Wang and Charles, 1991). Holmes (1948, 1955) found that he could eradicate spotted wilt disease from dahlia plants by rooting tip cuttings in soil. Murakishi and Carlson (1979) cultured *Brassica* initially infected with Turnip Yellow Mosaic virus and obtained 20% of regenerants that were virus-free.

2.1.6 Micropropagation and tissue culture of *Eucalyptus* species

Research at many Forestry Divisions has concentrated on developing commercial micropropagation techniques for rapid multiplication of species like *Melaleuca*, *Acacia* and *Eucalyptus*. Further, work has focussed mainly on micropropagation via axillary bud proliferation as this is simple technique that requires a minimum laboratory and nursery facilities (Table 2.2). Several reports have described micropropagation from seedlings and adult plants of *Eucalyptus* clones and hybrids (Barker *et al.*, 1977; De Fossard *et al.*, 1977; De Fossard, 1978; Lakshmi Sita and Vadyanathan, 1979; Hartney and Barker, 1980; Gupta *et* al., 1981; Gupta and Mascarenhas, 1983a; Das and Mitra, 1990; Le Roux and van Staden, 1991; Watt *et al.*, 1991) (Table 2.3). Two approaches have been used to propagate from nodal cultures, one being to produce multiple buds and shoots in aseptic cultures and then induce them to form roots (De Fossard and Bourne, 1977; De Fossard *et al.*, 1977). The other approach is direct induction of roots and shoots on an initial nodal explant

(Cresswell and De Fossard, 1974; De Fossard *et* al., 1974b; Cresswell and Nistch, 1975; Rao, 1988; Das and Mitra, 1990). Bachelard and Stowe (1962) were able to maintain cultures of seedling roots in a liquid medium containing 14% coconut milk. Root tips could be subcultured on the same medium, but no shoot regeneration was achieved. A study by Adam (1987) reported the possible isolation of roots on solid or liquid Murashige and Skoog (1962) medium without plant growth regulators. The study reported on genetic transformations of roots of *E. gunnii in vitro*. Culture of nodes and shoot tips have been attempted also. Rooted microcuttings were achieved with nodes of *E. grandis* up to node number 80 with the cotyledonary node taken as the first node (Cresswell and Nitsch, 1975), nodes from the crown of 5-year-old trees of *E. dalrympleana*, leaf discs of five-year old *E. macarthurii* trees (Durand-Cresswell and Nitsch, 1977), and from crowns of 25-year-old *E. ficifolia* trees (Baker *et al.*, 1977; De Fossard, 1981). However, there are reported problems such as the high level of explant contamination and extensive labour of mature tree crowns (Le Roux, 1991). This makes the shoot multiplication technique a better option left for researchers to exploit.

Le Roux (1991) obtained a large level of success in micropropagation of *Eucalyptus* species by the technique of inducing axillary and/or adventitious shoot proliferation on nodal explants, followed by rooting shoots. Early reports dating back to the late 70s and early 80s described the use of nodal explants from seedlings (De Fossard and Bourne, 1976, 1977; Hartney and Baker, 1980) but those extended to include those from mature trees (Mascarenhas *et al.*, 1982; Gupta *et al.*, 1983; Rao and Venkateswara, 1985), mature shoots grafted on seedling rootstocks (Durand-

Table 2.2: Some of the reports of studies on the micropropagation of *Eucalyptus* species via axillary bud proliferation/shoot culture (Adapted from George, 1996).

Species name	Type of culture	Source of explant	Results	References
E. bridgesiana	Node and callus	Node of 5-year-old tree	Axillary shoot formation. Semi-differentiated callus	Durand-Cresswell and Nistch (1977)
E. camaldulensis	Calloid	Shoot tips	Tissue subcultured. Plant regeneration	Boxus *et al.* (1991)
E. camaldulensis	Shoot	Node of mature tree	Shoot formation, proliferation and elongation	Gupta *et al.* (1983)
E. camaldulensis	Shoot	Nodal segments. ex seedlings or coppices	Multiple shoots. Rooted plants	Hartney (1982; 1983)
E. citriodora	Direct embryogenesis	Decoated mature seeds	Plantlets regenerated from germinated embryos	Muralidharan *et al.* (1989)
E. citriodora	Shoot	Shoot apices	Multiple shoots	Grewal *et al.* (1980)
E. citriodora	Shoot	Vegetative buds of 20-y. old trees	Axillary bud proliferation. Direct rooting	Gupta *et al.* (1981)
E. ficifolia	Shoot	Seedling node	Multiple bud formation. Direct rooting	De Fossard (1981)
E. globulus	Shoot	Terminal buds (10-20 y. old tree)	Axillary shoot proliferation and elongation. Rooted	Mascarenhas *et al.* (1982a)
E. grandis	Nodular callus	Hypocotyl of young seedlings	Multiple shoots over 3 yrs. Rooted plants	Warrag *et al.* (1991)
E. grandis	Shoot	Young shoots	Axillary shoot proliferation and elongation. Rooted	Sankara and Venkateswara (1985)
E. grandis	Shoot	Nodes ex 5-y.-old trees	Multiple shoots. Rooted	Warrag *et al.* (1989)
E. gunnii	Shoot	Not given	Shoot multiplication	Damiano *et al.* (1987)
E. gunnii	Shoot	Nodes from sterile seedlings	Shoots multiplied, elongated in dark. Rooted plants	Lubrano (1988)
E. marcarthurii	Shoot	Nodal explants ex seedlings and clonal hedges	Shoot proliferation. Rooted shoots to field	Le Roux and Van Staden (1991)
E. marginata	Shoot	Shoot tips of mature tree	Shoot multiplication. Direct rooting	McComb and Bennett (1982)
E. tereticornis	Direct	Seedling hypocotyl segments	Multiple shoots regenerated, rooted	Subbaiah and Minocha (1990)
E. teretocornis	Shoot	Nodes	Bud multiplication and elongation. Shoots rooted	Das and Mitra (1990)
E. teretocornis	Shoot	Axillary buds on mature stem nodes	Multiple shoots. Rooted	Rao (1988)
E. viminalis	Shoot	Shoot tips, nodal stem	Adventitious bud proliferation; shoot growth. Rooted	Mehra-Palta (1982)
E. viminalis	Shoot	Nodes from sterile seedlings	Shoots multiplied, elongated in dark. Rooted plants	Lubrano (1988)
E. grandis	Shoot	Seed	Shoots multiplied, elongated in dark. Rooted plants	Watt *et al* (1996)

Cresswell *et al.*, 1982; Franclet and Boulay, 1982; Boulay, 1983) and from coppice (Hartney, 1982a, 1982b; Burger, 1987).

In *in vitro* nodal cultures, new buds and shoots continue to arise in the axils of leaves and of new shoots, often without elongation, resulting in dense clumps of buds (Barker *et al.*, 1977). This process is usually accompanied by the development of some callus at the base of the explant, where it is in contact with the medium (Barker *et al.*, 1977). Various agar based media have been used to stimulate shoot multiplication, usually with a low auxin/cytokinin ratio (Le Roux, 1991). Liquid media were used to stimulate axillary shoot proliferation from buds of 20-year-old *E. citriodora* trees (Gupta *et al.*, 1981) and from nodes of *E. tereticornis* trees (Mascarenhas *et al.*, 1982). Multiplication rates achieved have varied and depend on species, clone, explant source and juvenility. High multiplication rates have been reported; for example, *E. maginata* seedling clones showed multiplication rates between three-fold and twenty-fold during four-week subculture periods (Bennett and McComb, 1982).

As previously mentioned, an alternative route of micropropagation is somatic embryogenesis which offers several advantages as a cheap large scale plant production system (Ammirato, 1983; Boulay, 1987). Somatic embryogenesis has been obtained in several tree species including *Eucalyptus* spp. (Table 2.3). Muhalidharan and Mascarenhas (1987) reported primary somatic embryogenesis on embryos of *E. citriodora* on semi-solid agar based B5 medium (Gamborg *et al.*, 1968) with 3 mg/l NAA, and 50 mg/l sucrose. Somatic embryos only developed on

embryos which had been soaked in sterile distilled water at 29 °C for two days prior to placement on medium. Qin Chang-Le and Kirby (1990) induced embryo-like structures in cultures of hypocotyls, cotyledons, and young seedling leaves of *E. botryoides*, *E. dunnii*, *E. grandis* and *E. rudis*, and also from young leaves of cultured shoots of superior adult clones of *E. grandis*.

Suspension cultures have been established by many researchers as another means of micropropagation. Sussex (1965) established friable callus from *E. camaldulensis* hypocotyls on White (1943) medium using 1 mg/l 2,4-D and 15% coconut milk. That author went further to establish suspension cultures from friable callus in an agitated liquid medium with the same growth regulators. Aggregates that were nodular in form resulted from single cells in liquid culture, and these could be established as friable callus on agar medium. Friable callus of *E. gunnii* and *E. gunnii* x *E. dalrympleana* hybrid was established from leaves on Murashige and Skoog (1962) medium with 2 mg/l 2,4-D (Teuliers *et al.*, 1989). Suspension cultures have been initiated to provide a potential source for protoplasts of *E. grandis*, *E. saligna* and *E. urophylla* for physiological and genetic studies (Dos Santos *et al.*, 1990; Penchel and Kirby, 1990a; 1990b; Watt *et al.*, 1992).

Protoplasts, in contrast to complete cells, are known to show differences in resistance to low temperature and their viability was always correlated with the degree of frost resistance of the mother tree (Le Roux, 1991). Teulieres *et al* (1989) suggest the use of this technique for the selection and propagation of frost resistant variants. Protoplasts of *E. grandis* have been isolated and cultured from hypocotyls,

Table 2.3: Summary of some of the reported studies on somatic embryogenesis of *Eucalyptus* species. This table summarises the conditions used and includes species name (column 1), explant type represents the starting material (column 2), regeneration route in column 3 indicates direct (DSE) or indirect (ISE) somatic embryogenesis, direct shooting (DS) and plantlet development (PI). Final structures resulted are indicated in column 4, medium type is represented in column 5,6,7 and 8, and references used in column 9. Media are solid unless specified to be in liquid form (L), and modified vitamins and amino acids which have been taken from a source other than that of the authors are also pointed out. (Adapted from Le Roux and van Staden, 1991, and Blakeway 1992).

Species	Explant	Regenerative route	Final structures	Medium	Nutrient modifications	Sucrose (gl^{-1})	Growth regulators	Reference
E. botryoides	hypocotyls, cotyledons, leaves,	DSE	embryo-like structures	MS	modified salts, RV vitamins and amino acids		(1.1) 2,4-D	Qin Chang-Le and Kirby (1990)
	embryo-like structures	DSE, DS	embryo-like structures	MS			(1.1) BA	
E. citriodora	embryos	DSE	early embryogenic	Gamborg		50	(3) NAA	Mualidharan and
	embryoids	DSE	stages embryogenic callus	B5 Sussex,	modified salts, glycine	50	(0.5) BA, (10 %) CM	Mascarenhas (1987)
	embryos	DSE-PI	plantlets	MS vitamins (MS-half strength		20	none	
E. citriodora	seeds (coat removed)	G-DSE	germinated seeds, embryo genesis on	Gamborg B5		50	(3) NAA	Muhalidharan, Gupta and
		DSE	cotyledons			50	(3) NAA	Mascarenhas
	embryos	DSE	embryo clusters	Gamborg	500 gl^{-1} glutamine	30	(5) NAA,	(1989)
	embryos	SE-PI	embryogenic mass	B5		20	(500) CH	
	embryos		plant development	Gamborg B5 Gamborg B5			none	
E. dunnii	hypocotyls, cotyledons,	DSE	embryo-like structures	MS	modified salts, RV vitamins, and		(1.1) 2,4-D	Qin Chang-Le and Kirby (1990)
	leaves	DSE, DS		MS	amino acids		(1.1) BA	
	Embryo-like		embryo-like		modified salts, RV			
	strutures	DSE, DS	structures, adventitious shoots	MS	vitamins, and amino acids		(1.1) BA	
	Embryo-like structures		embryo-like structures, adventitious shoots		modified salts, RV vitamins, and amino acids			
E. grandis	nodes (4-y-old trees)		axillary shoots	MS	800 mgl^{-1} PVP	20	(0.1-1)	Lakshmi Sita,
	shoots		callus	MS	800 mgl^{-1} PVP		NAA, (0.5-1) BA	Rani and Sankara
	callus	ISE	embryogenic callus	MS	800 mgl^{-1} PVP		(5) 2,4-D	Rao (1986)
	callus	ISE	embryogenic structures	MS (L)			(0.1) NAA, (5) KIN	
							(1) NAA, (1) BA	
E. grandis	hypocotyls, cotyledons, leaves, young leaves from	DSE	embryo-like structures	MS	modified salts, RV vitamins, and amino acids		(1.1) 2,4-D	Qin Chang-Le and Kirby (1990)
	shoots of adult trees	DSE, DS		MS			(1.1) BA	
	embryo-like structures		embryo-like structures, adventitious shoots		modified salts, RV vitamins, and amino acids			
E. grandis	leaves	ISE	embryogenic callus	MS		30	(0.25-1) 2,4-D	Watt, Blakeway,
	embryogenic callus	ISE	plantlets	MS	(4 gl^{-1}) activated charcoal	30	(0.01) NAA, (0.1) BA, (0.1) GA_3	Cresswell and Herman (1991)

cotyledons, embryo's hypocotyl derived from callus and cell suspensions, internodes from young shoots of adult trees, and from young leaves of *in vitro* grown shoots of adult trees (Penchel and Kirby, 1990a, 1990b).

In 1991, Le Roux macropropagated E. *macarthurii*, *E. smithii*, *E. grandis* and *E. saligna* by cuttings. However, no other work has been reported on micropropagation of cold-tolerant *Eucalyptus* clones of interest to the South African Forestry Industry.

2.1.7 Aims of this investigation

The potential exists to use the same techniques discussed above for non-cold tolerant *Eucalyptus*, to optimize protocols for the micropropagation of cold-tolerant clones. The aim of this investigation was, therefore, to address this deficiency. Studies on different *Eucalyptus* species and hybrids have shown that precise methodology must be established for each clone, variety, explant type and culture system. The strategy employed was to adapt and optimize established protocols for subtropical *Eucalyptus* clones and hybrids for axillary bud proliferation (Watt *et* al., 1992; 1996) and somatic embryogenesis (Watt *et al.*, 1991) to three cold-tolerant *E. saligna* (AS 196) and *E. saligna* x *grandis* (PG 28 and PG 47).

2.2 MATERIALS AND METHODS

2.2.1 Plant material

a) For axillary bud proliferation

Branches of three cold-tolerant clones of *Eucalyptus* spp., *E. saligna* (AS 196) selected in Australia and *E. grandis* x *saligna* (PG 47 and PG 63) (Mondi Forests, SA), were cut from field grown plants in clonal hedges. Harvested shoots were then kept in a sealed plastic bags for 1- 2 h in 1 gl^{-1} Benlate® (Effecto, SA) + 1 gl^{-1} boric acid + 0.5 mll^{-1} Bravo® [Shell, S.A (Pty) Limited] fungicide. Stems approximately 250 to 350 mm long were cut from the branches and were immediately rinsed well in running water. The leaves were trimmed such that approximately two thirds of each leaf was removed.

b) For callus production

Leaves of the same clones as used for axillary bud proliferation were collected and kept in sealed plastic bags as described in the above section.

2.2.2 Sterilization of explants

a) Bud/branches

After harvest and initial preparation (2.2.1.a), shoots were transferred into 1 l Schott bottles containing 500 ml sterile water after which, water was poured out and 0.02 % (w/v) mercuric

chloride and a drop polyoxyethylene sorbitan monolaurate (Tween 20) were added. Shoots were allowed to soak in for 2 min. The mercuric chloride solution was poured out and the shoots were rinsed three times with sterile water. Then, 1 % (w/v) calcium hypochlorite was added and the shoots were soaked for 2 min. and then washed free from the sterilant three times. Then shoots were ready to be plated onto shoot initiation medium.

b) Leaves

Leaves were sterilized as described in section 2.2.2.a above. Lastly, the leaves were lastly trimmed (edges cut transversely) and stabbed three-four times with a scalpel blade (Lainé and David, 1994) prior to culture and placed, adaxial face up, onto callus induction medium.

2.2.3 Culture manipulations for axillary bud proliferation

A schematic representation of the protocol for axillary bud proliferation is shown in Fig. 2.3.

a) Bud induction

Sterilized shoots were cultured in glass tubes containing 10 ml of bud induction medium containing Murashige and Skoog nutrients (MS), 0.1 mgl^{-1} biotin, 0.1 mgl^{-1} calcium pantothenate, 0.04 mgl^{-1} 1-naphthylacetic acid (NAA), 0.11 mgl^{-1} benzyl amino purine (BAP), 6-furfurylaminopurine (kinetin) (FAP), 20 gl^{-1} sucrose, pH 5.8. The effect of gelling agents on bud

break was investigated by using one of the following: 3.5 gl^{-1} Gelrite, 10 gl^{-1} Saarchem agar (Unilab, S.A.), 1.75 g/l Gelrite + 5 g/l agar, 6 g/l Kalys agar or vermiculite (Fig. 2.4). The bud induction stage involved two passages of two weeks each and the different media were tested in both. Further, omission of stage I from the protocol was tested. Tubes were kept in the culture room under a 16 h photoperiod at 200 $\mu E.M^{-2}.s^{-1}$ photosynthetic photon flux density (PPFD) for a period of one-two weeks at 24 ± 2 °C.

b) Shoot multiplication

For the multiplication phase experiments, the following two auxins were tested: BAP and NAA ranging in concentration from 0.01 to 0.5 mgl^{-1} (w/v). All treatments had MS nutrients supplemented with 0.1 mgl^{-1} biotin, 0.1 mgl^{-1} calcium pantothenate, 0.01 mgl^{-1} NAA, 0.2 mgl^{-1} BAP, 20 gl^{-1} sucrose and 3.5 gl^{-1} Gelrite, pH 5.8, in common (passage I). After 2 weeks, buds were again transferred onto the same three media (passage II). These experiments were repeated three to twelve times and routine subcultures were carried out in glass bottles with three to five explants per bottle. The cultures were maintained at 24 ± 2 °C under a 16 h photoperiod at 200 $\mu E.m^{-2}.s^{-1}$ photosynthetic photon flux density (PPFD) for two weeks. At the end of a subculture, variables such as the number of shoots produced per explant, the number of explants showing senescence, contamination and multiplication rates, were recorded.

c) Shoot elongation

The explant shoots were removed from the multiplication medium and transferred to shoot elongation media. These comprised of MS nutrients supplemented with, 0.1 mgl^{-1} biotin, 0.1 mgl^{-1} calcium pantothenate, 20 gl^{-1} sucrose and 3.5 gl^{-1} Gelrite, pH 5.8, and various growth regulator combinations. These were NAA and IBA [0.05 to 0.35 mgl^{-1} (w/v)] tested in various combinations with FAP [0.1 to 0.2 mgl^{-1} (w/v)]. Contamination, senescence, and length of explants, were recorded. Cultures were maintained at 24 ± 2 °C under a 16 h photoperiod at 200 $\mu E.m^{-2}.s^{-1}$ photosynthetic photon flux density (PPFD) for four weeks.

d) Root induction

Root induction was attempted with suitable shoots at the end of elongation subculture. Suitable shoots for root induction were considered as those which had elongated to a length of 10 to 20 mm and which had healthy, green, and slightly expanded leaves. The lower leaves on the explants were trimmed close to the stem to avoid the formation of callus on the leaf surfaces due to contact with the medium (Le Roux, 1991).

Individual shoots were subcultured onto rooting media supplemented with half-strength MS nutrients and two auxins, NAA and IBA ranging in concentration from 0.1 to 1 mgl^{-1} (w/v).

Cultures were maintained at 24 ± 2 °C under a 16h photoperiod at 200 $\mu E.m^{-2}.s^{-1}$ photosynthetic photon flux density (PPFD) for four weeks.

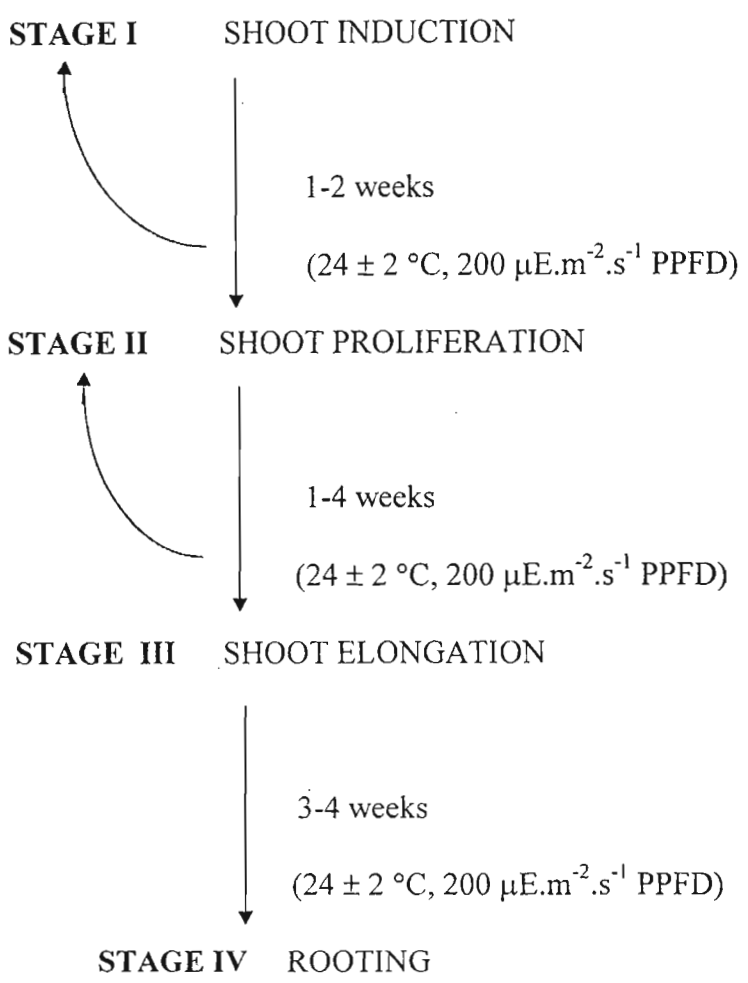


Figure 2.3: Diagrammatic representation of the culture stages involved in the production of plantlets via axillary bud proliferation.

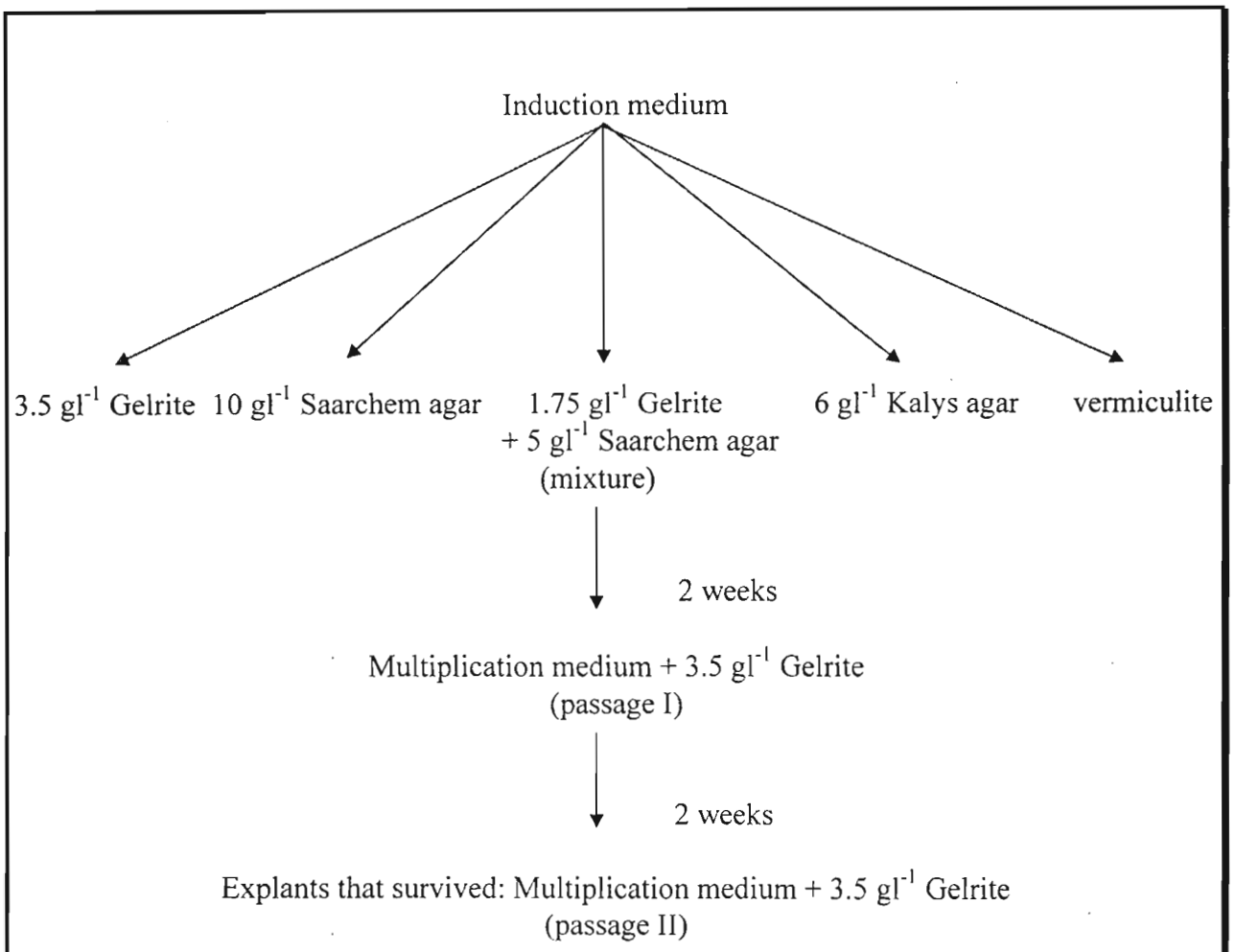


Figure 2.4: Protocol to investigate the effect of gelling agents during stage I (on Induction medium).

2.2.4 Culture conditions for callus induction

a) Establishment of the optimum callus induction medium

A number of different callus induction medium (CIM) were tested (Table 2.2). These media contained MS nutrients with varied concentrations of sucrose and one of two different gelling agents, *viz.* 4 gl^{-1} Gelrite or 10 gl^{-1} Saarchem agar, pH 5.8. Leaf segments (0.5 mm x 0.5 mm) were used to induce callus (Blakeway, 1992). These cultures were maintained at 24 ± 2 °C under a 16h photoperiod at 200 $\mu E.m^{-2}.s^{-1}$ photosynthetic photon flux density (PPFD) for four weeks. Percentage explants with callus, extent of contamination and senescence, were recorded. High levels of contamination were observed that resulted in the planning of the eradication of endogenous contaminants experiments, as described in the following sections.

b) Establishment of the optimal sterilization treatment for removal of endogenous contaminants

The complete loss of cultures due to microbial contamination when explants were taken from mature trees established an urgent problem that had to be solved relating to disinfection of explants. A schematic representation of the first sterilization treatments is shown in Fig. 2.5. Half of the leaf material was soaked overnight in one of two different liquid media. The first medium was supplemented with antibiotic cocktail 1 [20 μgml^{-1} Rifampicin® (Boehringer, Mannheim) + 30 μgml^{-1} Trimethoprin® (Boehringer, Mannheim)] and 2 mgl^{-1} Previcur®

(Schering AG, Pflanzenshutz, Berlin, Germany) whereas the second one had antibiotic cocktail 2 [100 μgml^{-1} Rifampicin® + 100 μgml^{-1} Streptomycin® (Boehringer, Mannheim)] and 2 mll^{-1} Previcur® (Fig. 2.5). The remaining half was plated directly either on CIM (MS nutrients supplemented with 0.1 mgl^{-1} biotin, 0.1 mgl^{-1} calcium pantothenate, 0.01 mgl^{-1} NAA, 0.2 mgl^{-1} BAP, 30 gl^{-1} sucrose, 1 mgl^{-1} 2,4-D and 4 gl^{-1} Gelrite, pH 5.8) supplemented with antibiotic cocktail 1 and 2 mll^{-1} Previcur® or CIM with antibiotic cocktail 2 and 2 mll^{-1} Previcur®. For those washed in liquid medium with antibiotic cocktail 1, half of them were plated on CIM without any antibiotic cocktail and the other half was plated on CIM with antibiotic cocktail 1 and 2 mll^{-1} Previcur®. Similarly, for those that were washed in liquid medium with antibiotic cocktail 2 and 2 mll^{-1} Previcur®, half the number was plated on CIM devoid of any antibiotic cocktail and Previcur®, while the other remaining half was plated on CIM with antibiotic cocktail 2 and 2 mll^{-1} Previcur®.

In another investigation (Fig. 2.6), explants were washed overnight in the liquid medium comprised of ¼MS nutrients, 5 gl^{-1} sucrose and 0.2 mll^{-1} Previcur®. Soaked leaf discs were cultured the following morning onto the five CIM given in Fig. 2.4. All the media had MS nutrients, 30 gl^{-1} sucrose, 1 mgl^{-1} 2,4-D and 4 gl^{-1} Gelrite in common but with different supplements as depicted in Fig 2.6.

In the last investigation, explants were washed overnight in three different liquid media with different pH. The media were comprised as follows:

1) ¼MS + 5 gl^{-1} sucrose and 0.2 mgl^{-1} Previcur ® + 0.1 mll^{-1} antibiotic cocktail, pH 8.0.

2) ¼MS + 5 gl^{-1} sucrose + 1 gl^{-1} Benlate® + 1 mll^{-1} antibioitc cocktail 2, pH 5.6.

3) ¼MS + 5 gl^{-1} sucrose + 1 gl^{-1} Benlate® + 1 mll^{-1} antibiotic cocktail 2, pH 8.0.

The leaves were cultured in MS nutrients supplemented with 30 gl^{-1} sucrose, 1 mgl^{-1} 2,4-D and 1 gl^{-1} Benlate®. All these were incubated in the dark at 24 ± 2 °C for four weeks. Variables recorded were extent of contamination, senescence and percentage explants with callus.

Table 2.2: Composition of media used for the induction of calli of clones AS 196, PG 28 and PG 47. All treatments contained full MS (Murashige and Skoog, 1962) nutrients and 30 gl^{-1} sucrose. Gelling agent and concentration of the plant growth-regulator, 2,4-dichlorophenoxyacetic acid (2,4-D), were varied as indicated.

Medium code	Gelling agent (gl^{-1})	2,4-D (mgl^{-1})
CIM 1	Gelrite (4)	1
CIM 2	Gelrite (4)	3
CIM 3	Saarchem agar (10)	1
CIM 4	Saarchem agar (10)	3

2.2.5 Microscopy and photography

The various stages of bud induction, multiplication, elongation and rooting were recorded using a Nikon FM2 camera with a 60 mm Mikro Nikkor macro lens. The calli were examined microscopically using a Wild M3 stereomicroscope and photographically recorded using a Wild Photoautomat MPS 55 system and a Nikon FX 35A and UFX photorecording system. The presence of embryogenic cells was also recorded.

2.2.6 Analysis of data

Average values were calculated from the data recorded during different stages of bud induction, shoot multiplication, shoot elongation, rooting and callus production via somatic embryogenesis. Where appropriate, One-Way Analysis of Variance (ANOVA) (SAS, 1987) was used to assess differences in the recorded mean values of the variables investigated. Alphabetical values were assigned to the mean values recorded for each treatment. Mean values that did not share the same letter, were recognised as being significantly different from each other.

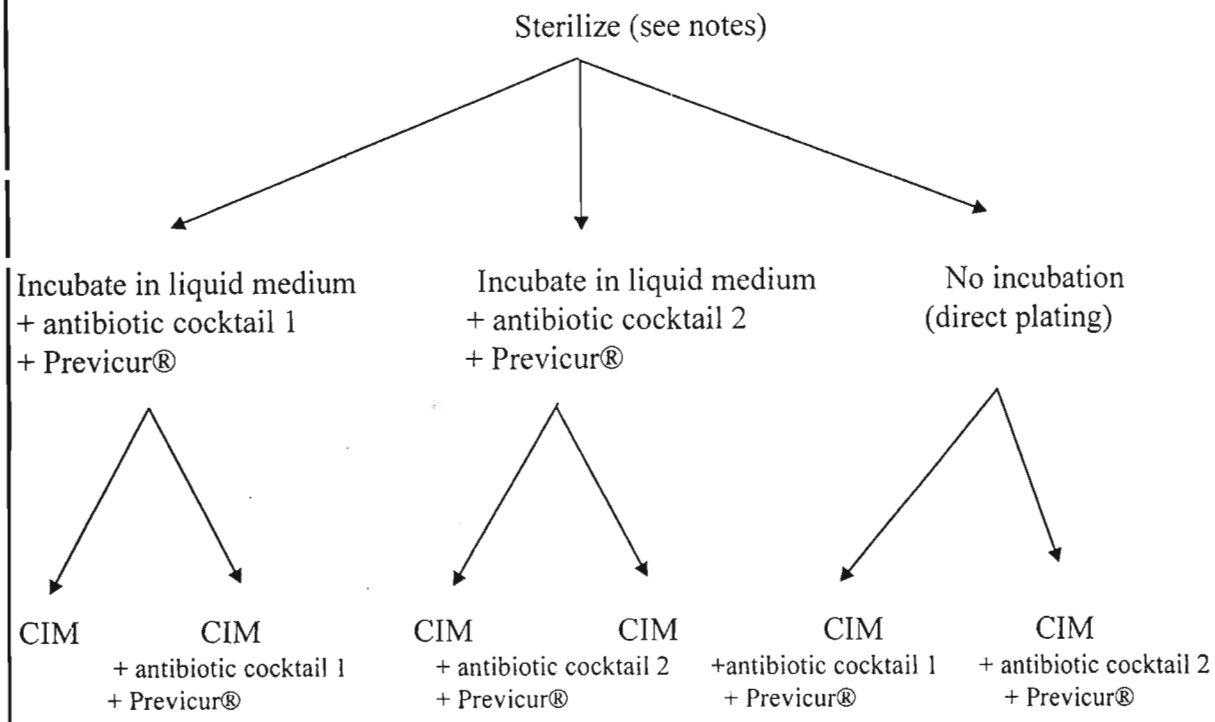


Figure 2.5: Schematic representation of a protocol for eradication of fungal and bacterial contaminants. The liquid medium for incubation was made as follows: ¼MS + 5 gl^{-1} sucrose + 0.2 $ml l^{-1}$ Previcur®. Cocktail 1 = 20 $\mu g ml^{-1}$ Rifampicin® + 30 $\mu g ml^{-1}$ Trimethoprin® whereas Cocktail 2 = 100 $\mu g ml^{-1}$ Rifampicin® + 100 $\mu g ml^{-1}$ Streptomycin®. Previcur® = 2 $mg l^{-1}$.

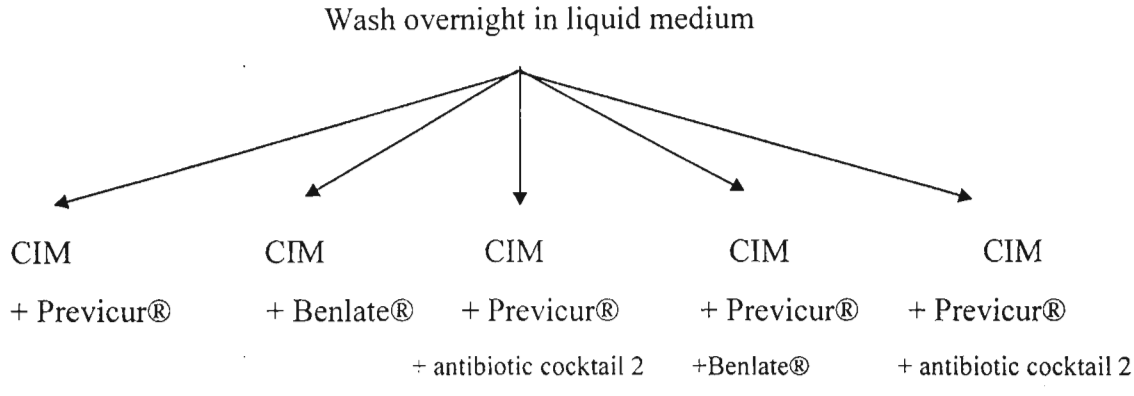


Figure 2.6: Diagrammatic representation of a method adopted after the first outline in Figure 2.5. Media as described in Fig. 2.5 and Benlate® = 1 gl^{-1}.

2.3 RESULTS AND DISCUSSION

2.3.1 Plantlet production via axillary bud induction

a) The effect of some culture conditions on axillary bud induction from mature plants

For every stage, appropriate parameters were tested to improve yield. Sterilized buds of three cold-tolerant clones of *Eucalyptus* spp., *E. saligna* (AS 196), *E. grandis* x *saligna* (PG 28 and PG 47) were placed in culture tubes on different bud induction media consisting of MS nutrients supplemented with 0.1 mgl^{-1} biotin, 0.1 mgl^{-1} calcium pantothenate, 0.01 mgl^{-1} NAA, 0.2 mgl^{-1} BAP. The media had five different gelling agents as will be discussed in section 2.3.1a*i* (Table 2.4). Buds were then placed in the light for about one week (Plate 2.1).

i) Effect of gelling agent on bud induction

The effect of gelling agents during stage I on bud induction medium was assessed only after buds were in the second transfer of bud multiplication medium because the principal aim was to observe their extended effect in bud multiplication. Multiplication occurred only during the second passage on multiplication medium consisting of MS nutrients supplemented with 0.1 mgl^{-1} biotin, 0.1 mgl^{-1} calcium pantothenate, 0.01 mgl^{-1} NAA, 0.2 mgl^{-1} BAP, 20 gl^{-1} sucrose and 3.5 gl^{-1} Gelrite (Table 2.3). However, most initial explants had died during the first two weeks of the first passage on multiplication medium gelled with 3.5 g/l Gelrite. Of those that survived the two passages, the average number of shoots per explant was only approximately three for *E. saligna* x *grandis* (PG

63) and 4 to 6 shoots per explant for *E. saligna* (AS 196) (Table 2.3). These survived the transfer into elongation medium. Although vermiculite gave comparatively good survival rates, it was omitted from further work as it was difficult to prevent it from drying out. Jay-Allemand *et al.* (1993) obtained much better results than on a semisolid medium when shoots were transferred into vermiculite wetted with gelled ¼ Driver and Kuniyuki (DKW) medium.

ii) Effect of gelling agents on shoot multiplication medium

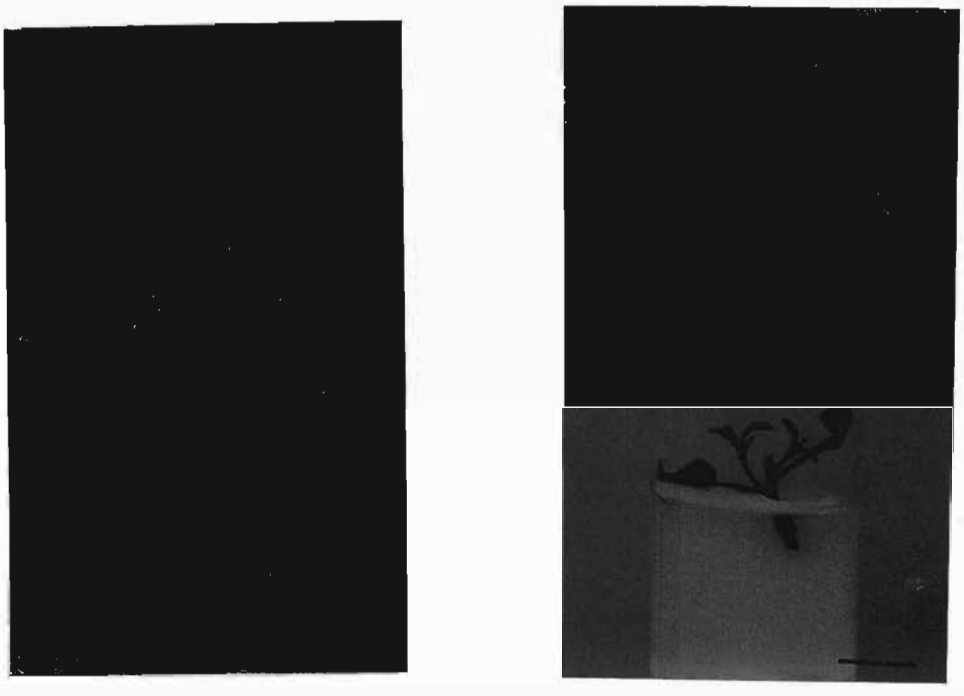
After buds were succesfully induced on bud induction medium supplemented with 10 g/l Saarchem agar and Kalys agar, with 80 % and 100 % survival respectively, the two were selected to be the gelling agents during this stage. The different gelling agents initially tested in bud induction stage were also tested in bud multiplication stage. The results featured *E. saligna* (AS 196) alone because of lack of material in other clones, and showed that in first passage on shoot multiplication medium from bud induction induction medium gelled with Saarchem agar, all the gelling agents gave almost equal survival percentages (91 %) with the mixture of Saarchem agar and Gelrite giving the lowest percentage survival (54 %) (Table 2.4). When Kalys agar was used during the first stage (bud induction), the results in the second passage showed that Saarchem agar and Gelrite were comparatively better (with 75 % and 100 % survival rates, respectively) than their mixture (50 %) (Table 2.4). However, when Saarchem agar was used during bud induction, the results showed that Saarchem agar and Kalys agar were comparatively better with 91% and 73% percentage survival (Table 2.4).

Table 2.3: Effect of gelling agent during bud induction of *E. saligna* (AS 196) and *E. saligna* x *grandis* (PG 63) on multiplication. Buds were induced in MS nutrients supplemented with 0.1 mgl^{-1} biotin, 0.1 mgl^{-1} calcium pantothenate, 0.01 mgl^{-1} NAA, 0.2 mgl^{-1} BAP and 20 gl^{-1} sucrose. Gelling agents used were 3.5 gl^{-1} Gelrite, 10 gl^{-1} Saarchem agar, 1.75 gl^{-1} Gelrite + 5 gl^{-1} Saarchem agar, 6 gl^{-1} Kalys agar and vermiculite, pH 5.8. Induction was recorded after approximately one week and buds weere then transferred to multiplication medium for two passages, on medium containing the same gelling agent as for bud induction. n= 50-100.

Treatment		1st passage		2nd passage		
Clone	Treatment	Contamination (%)	Survival (%)	Survival (%)	Explants with multiplication (%)	Average number shoots/ explant
AS 196	mixture	0	0	0	0	0
	Saarchem agar	0	80	80	80	2.5
	Gelrite	0	43	28	28	6
	vermiculite	0	60	33	33	4
	Kalys agar	0	100	100	100	3.8
PG 63	mixture	10	72	43	43	2.7
	Saarchem agar	7	91	50	50	2.8
	Gelrite	13	87	25	25	3
	vermiculite	10	80	40	40	2.7
	Kalys agar	not done	-	-	-	-

Table 2.4: Effect of gelling agents during stage II on multiplication medium. The gelling agents used in bud induction were 10 gl^{-1} Saarchem agar and 6 gl^{-1} Kalys agar. Data is for *E. saligna* (AS 196) only. n= 50-100.

Treatment		1st passage		2nd passage		
Gelling agent in bud induction	Gelling agent in multiplication	Survival (%)	Explants with multiplication (%)	Survival (%)	Explants with multiplication (%)	Average number shoots/ explant
Saarchem agar	mixture	54	0	54	54	2
	Saarchem agar	91	0	91	91	3
	Gelrite	91	0	64	64	3
	Kalys agar	91	0	73	73	3.3
Kalys agar	mixture	75	0	50	50	3
	Saarchem agar	75	0	75	75	3.3
	Gelrite	100	0	75	75	4
	Kalys agar	100	0	100	100	5



a) b)

Plate 2.1: Example of shoot production of *E. saligna* (AS 196) through axillary bud proliferation a) during initiation (bar = 5 mm)

b) and after one week in culture (bar = 7.1 mm)

Although, average shoots per explant in Gelrite (4 shoots/ explant) was slightly above that achieved in Saarchem agar (3.3 shoots/explant), Gelrite was regarded as the better gelling agent in second passage as it gave a better percentage survival rate. The results in Table 2.4 also showed that no multiplication occurred by the end of the first passage. The poor results at the end of the second passage are mainly due to death after two to three weeks of the first passage. In order to overcome this problem, the length of the first passage was reduced to two weeks after which the buds were removed from their main stem and transferred to fresh shoot multiplication medium. Since the results showed that buds multiplied better in medium gelled with Gelrite, the elongation medium was also gelled with the latter.

iii) Effect of growth regulators on shoot multiplication

In this experiment, the parameter to investigate was the effect of growth regulators on bud multiplication. Similar treatments were tested for all cold-tolerant clones mentioned in section 2.3.1a. Shoots which developed in bud induction medium multiplied by axillary bud development when transferred to shoot multiplication medium (Plate 2.2)(Table 2.5). Though the results were statistically insignificant amongst treatments in a clone, there were variations within treatment and medium M2 (MS nutrients supplemented with 0.1 mgl^{-1} biotin, 0.1 mgl^{-1} calcium pantothenate, 0.2 mgl^{-1} BAP, 20 gl^{-1} sucrose and 3.5 gl^{-1} Gelrite) proved to be the best for both *E. saligna* (AS 196) and *E. saligna* x *grandis* (PG 28 and PG 47) (Table 2.5). This medium was selected because of its highest percentage multiplication, 93 % and 95.4 %; average number of shoots per

explant (4 and 3.1) for both *E. saligna* (AS 196) and *E. saligna* x *grandis* (PG 47), respectively. This medium was also selected to be the best for clone *E. saligna* x *grandis* (PG 28) because its lowest multiplication rate (33.3 %) was compensated by the highest average number of shoots per explant (5.2). Examples of shoot multiplication cultures are illustrated in Plate 2.2. The results of this present study are supported by the findings of LakShimi Sita and Vaidyanathan (1979), who used MS basal medium supplemented with BAP alone at 0.5 and 2 mg/l concentrations. Those treatments showed that *E. citriodora* multiple shoot production occurred within 15 days compared with those of this present study. On basal medium supplemented with the lowest concentration of BAP (0.5 mg/l), ten shoots developed and on higher concentrations of BAP, the multiplication number increased.

In the present studies, there were problems that were experienced during at the multiplication stage. The first was the development of white sugary callus on leaf surfaces, stem nodes and in the axils of leaves (Plate 2.3). This problem has been reported for *E. dalrympleana*, *E. delagatensis* and *E. ficifolia* (De Fossard *et al.*, 1978; Boulay, 1983), and *E. dalrympleana* (Durand-Cresswell *et al.*, 1982). Durand-Cresswell *et al.* (1982) stated that the nodal callus begins its development from the abscission layers on the petiole. This callus, caused by the excision of stem from parent branch, often develops into a mass which overtakes the axillary bud, causes leaf drop and the senescence and abscission of the apical shoot and axillary branches. The second problem was the production of brown exudate produced by the explants and was accompanied by

the death of shoots (Plate 2.3). This observation is similar to the one made by Durand-Cresswell (1977) in shoots of *E. grandis*. Both Asahira and Nitsch (1968) and Rabechault *et al.* (1976) showed that cytokinins provoke browning and growing cultures in the dark has been suggested to remedy this problem.

iv) Elongation of shoots

The elongation phase is often necessary because it is difficult to root buds smaller than 10 mm (Boulay, 1985). In this study, explants from multiplication stage were excised singly and transferred onto elongation medium consisting of MS nutrients supplemented with 0.1 mgl^{-1} biotin, 0.1 mgl^{-1} calcium pantothenate, 20 gl^{-1} sucrose and 3.5 gl^{-1} Gelrite. Concentrations of various PGRs (NAA, FAP and IBA) ranged from 0.1 to 0.35 mgl^{-1}. The results of this study showed that elongation medium supplemented with 0.35 mgl^{-1} NAA, 0.1 mgl^{-1} FAP and 0.1 mgl^{-1} IBA gave relatively good results with *E. saligna* (AS 196), shoots growing to a length of 50 mm within six weeks (Table 2.6) (Plate 2.4). Contamination by both bacteria and fungi did not occur at all in any of the treatments which were investigated. This selected elongation medium supplemented with 0.35 mgl^{-1} NAA, 0.1 mg/l FAP and 0.1 mg/l IBA was also used to elongate clones of *E. saligna* x *grandis* (PG 28 and PG 47) and the results showed shoot elongation of about 40-50 mm. The elongated shoots were used for rooting experiments.

v) Rooting studies

Rooting percentages of shoots were disappointingly poor in almost all root induction treatments in both *E. saligna* (AS 196) and *E. saligna* x *grandis* (PG 28 and PG 47). A summary of rooting percentages of buds for all treatments is presented in Table 2.7. Best rooting medium for *E. saligna* x *grandis* (PG 28) and *E. saligna* (AS 196) *in vitro* (4 %) was achieved when using 1 mgl^{-1} IBA (Plate 2.5.a). Shoots of both *E. saligna* (AS 196) and *E. saligna* x *grandis* (PG 47) proved to be very difficult to root with almost 0 % success for all treatments tested. The results of this work are contrary to previous studies on *E. deglupta* (Paton *et al.*, 1970), *E. platyphylla* and *E. grandis* (Durand-Cresswell, 1977) reported that IBA induced root initiation (Durand-Cresswell, 1977). Further, in this study IBA was found also to cause exudate formation which often led to the death of shoot cultures (Plate 2.5.b). These poor results are similar to those reported by Bennett and McComb (1982) who obtained only 5 % rooting from nodal shoot cultures of mature trees of *E. marginata* after treatment with 2 mg/l IBA. The results of this current study suggested that either the culture conditions for root initiation in both *E. saligna* (AS 196) and *E. saligna* x *grandis* (PG 28 and PG 47) were not optimal or the shoots cultured were not in a state of physiological activity. Also, it appeared that either a complementary factor for root development was absent or other exogenous growth factors should be tested.

Gupta *et al.* (1983) showed that IBA stimulated root initiation in the dark compared to the light.

Table 2.5: The effect of growth regulators on shoot multiplication of cold-tolerant *E. saligna* (AS 196) and *E. grandis* x *saligna* (PG 28 and PG 47) clones. Explants were placed in culture for one week to induce bud break (Stage I) and then buds were transferred to fresh multiplication medium for two weeks (Stage II). Media consisted of MS nutrients supplemented with 0.1 mgl^{-1} biotin, 0.1 mgl^{-1} calcium pantothenate, 20 gl^{-1} sucrose and 3.5 gl^{-1} gelrite, pH 5.8. M1= 0.01 mgl^{-1} NAA + 0.5 mgl^{-1} BAP; M2= 0.2 mgl^{-1} BAP and M3= 0.5 mgl^{-1} BAP. Levels of significant difference (ANOVA) are given. Treatments with similar letters were not significantly different (P= 0.05). n= 50-100.

		Stage I			Stage II		
Clone	**Treatment**	**Bud break (%)**	**Contamination (%)**	**Senescence (%)**	**Survival (%)**	**Explants with multiplication (%)**	**Average number shoots/explant**
AS 196	M1	83.7	6.1	0	100	78.8a	3.0
	M2	83.7	0	5.6	83.3	93a	4.0
	M3	83.7	3.3	0	100	80.6a	2.0
PG 28	M1	82.3	0	40	60	50a	3.4
	M2	82.3	10	46.7	33.3	33.3a	5.2
	M3	82.3	10	43.3	40	40a	3.3
PG 47	M1	81.6	11.1	23	62.9	88.2a	2.2
	M2	81.6	11.1	7.4	81.5	95.4a	3.1
	M3	81.6	11.1	22.2	70.4	73.7a	2.5

a) b)

Plate 2.2: Example of shoot multiplication in *E. saligna* (AS 196) through axillary bud proliferation a) during initiation (bar = 12.5 mm).

b) after four week in culture (bar = 13.5 mm).

Plate 2.3: Shoot culture of *E. saligna* x *grandis* (PG28) showing callus development on leaves and axillary buds (bar = 14.8 mm).

Table 2.6: Effect of growth regulators on shoot elongation of cold-tolerant *E. saligna* clone AS 196 after 4 weeks. Media consisted of MS nutrients, 0.1 mgl^{-1} biotin, 0.1 mgl^{-1} calcium pantothenate, 20 gl^{-1} sucrose and 3.5 gl^{-1} Gelrite, pH 5.8 and combinations of plant growth regulators (PGR), n= 30.

PGR (mgl^{-1})	Contamination (%)	Senescence (%)	Shoot length (mm)
NAA (0.35) FAP (0.1) IBA (0.1)	0	0	50
NAA (0.35) FAP (0.2) IBA (0.05)	0	0	30
NAA (0.35) FAP (0.2) IBA (0.1)	0	0	40

Table 2.7: Summary of results for rooting of *E. saligna* (AS 196) and *E. grandis* x *saligna* (PG 28 and PG 47) buds in response to PGR treatments. Buds from elongation medium were rooted in medium consisting of ½MS nutrients, 15 gl^{-1} sucrose and 3.5 gl^{-1} Gelrite, pH 5.8 and vermiculite where specified. Results were recorded after 4 weeks in culture. For a medium designated light, the cultures were put in the light until roots were developed and for those designated dark, cultures were initially put in the dark for a period of at least 7 days and then transferred to light conditons. n=15-30.

Clone	PGR (mgl^{-1})	Contamination (%)	Senescence (%)	Explants callused (%)	Light/Dark	Rooted (%)
AS 196	IBA (0.1)	20	44	100	Light	4
	IBA (0.5)	8	72	100	Light	0
	IBA (1)	25	20	100	Light	0
	NAA (0.5) + IBA (0.1)	25	20	100	Light	0
	Ascorbic(1) + Citric acid(1) + vermiculite	6.7	26.7	0	Light	0
	Ascorbic acid (1) + Citric acid(1)	20	53.3	0	Light	0
	Ascorbic acid (1) + Citric acid(1)	7.1	21.4	0	Dark	0
PG 28	NAA (0.5) + IBA (0.1)	8	48	100	Light	4
PG 47	IBA (1)	48	68	100	Light	0

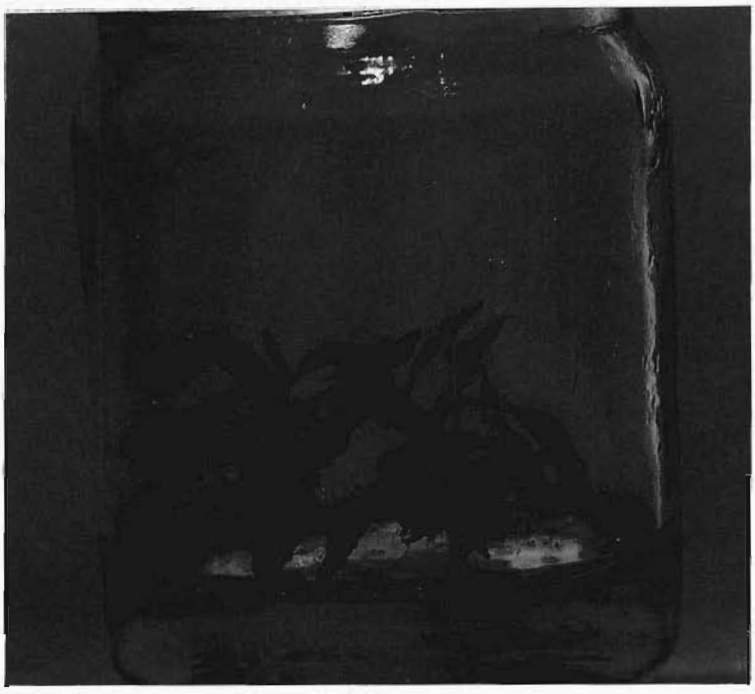
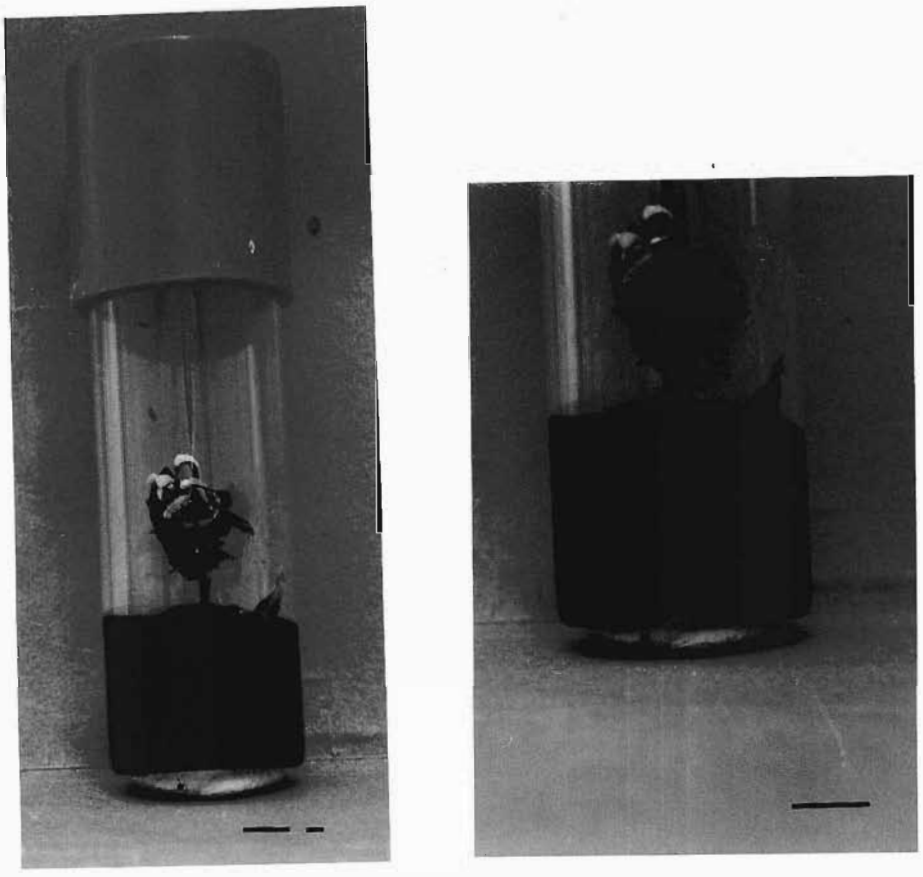


Plate 2.4: Shoot elongation of *E. saligna* (AS 196) on elongation medium after six weeks in culture (bar = 4.7 mm).



a) b)

Plate 2.5: Shoots in rooting medium of *E. saligna* x *grandis* (PG 28)

a) rooted shoot after four weeks in rooting medium supplemented with 1 gl^{-1} IBA (bar = 10 mm).

b) exudate and callus (bar = 7 mm).

The dark treatment was aimed at improving the rooting ability of shoots. Ascorbic acid has been shown to promote rooting of cuttings (Wareing, 1982). It is thought that it acts by inhibiting the effects of natural gibberellins which are inhibitory to rooting in some species and is also inhibitory to callus growth. Therefore, treatments using rooting medium supplemented with 1 mgl^{-1} ascorbic acid and 3.5 gl^{-1} Gelrite or with 1 mgl^{-1} ascorbic acid with vermiculite as a solidifying agent, were also tested both in light and in the dark. Results showed that the dark treatment prevents the leaching of brown substances but this treatment gave 0 % rooting (Table 2.7).

2.3.2 Initial studies on micropropagation via somatic embryogenesis

Sterilized leaf pieces of both *E. saligna* (AS 196) and *E. saligna* x *grandis* (PG 28 and PG 47) were placed on callus induction medium consisting of MS nutrients supplemented with 1 mgl^{-1} 2,4-D, 30 gl^{-1} sucrose and 4 gl^{-1} Gelrite (section 2.2.4.b) in order to induce callus. However, in initial investigations it was found that there was severe microbial contamination by both bacteria and fungi. This contamination problem greatly retarded experimentation aimed at determining cultural conditions suitable for the growth and development of callus.

a) Eradication of endogenous contaminants

Because of the contamination, a number of strategies were attempted in order to solve the contamination problem. The first involved the devicing of a decontamination protocol that would maximally eradicate endogenous contaminants. It aimed investigating better

methods of disinfecting field-grown adult material. The second involved the use of aseptic seedling material to gain information about suitable cultural conditions for the selected clones.

The surfaces of explants collected from stock plants growing in the field and in the glasshouse carry a microbial flora. Although not harmful to the plants, the microbial flora multiplies rapidly on the nutritive chemically defined culture medium, modifying the composition of the medium, and producing conditions that cannot be repeated (De Fossard, 1962; Cresswell and De Fossard, 1974). The problems of microbial contamination in organ culture work requires the worker to find disinfectant which is capable of killing all of the microbes on the excised organ without any phytotoxicity (Cresswell and De Fossard, 1974). In this present study, development of a reliable sterilization method was investigated. Surface-sterilized leaves of *E. saligna* (AS 196)(section 2.2.2) were treated with several disinfection treatments. ANOVA of the number of explants contaminated by both bacteria and fungi in the first experiment conducted showed no significant variation among the washings from leaves treated with antibiotic cocktails at p=0.05 (Table 2.8). Contamination was by both bacteria (8.2%-37.5%) and fungi (35%- 58.3%). ANOVA of explants contaminated with bacteria, in all leaf washing treatments showed no significant differences (p= 0.05). Bacterial contamination was lowest (8.2%) when explants were washed in antibiotic cocktail 1 and highest (37.5%) when explants were not washed in any antibiotic cocktail but rather

plated directly on callus induction medium supplemented with 2 mll^{-1} Previcur® and antibiotic cocktail 2. ANOVA of explants contaminated with fungi also showed differences among the leaf washing antibiotic treatments (p= 0.05) (Table 2.8). Fungal contamination was lowest when explants were not initially washed in any antibiotic cocktail treatment (35%) and highest (58.3%) when explants were washed in antibiotic cocktail 2. The results showed that explants were affected by the level of contamination by both bacteria and fungi. As a result, another investigation was conducted aiming at developing the most effect protocol for elimination of these endogenous contaminants. In this investigation, all leaf explants were washed in liquid medium ½MS supplemented with 5 gl^{-1} sucrose and 0.2 mll^{-1} Previcur®. ANOVA of the explants contaminated with bacteria showed significant differences among the callus induction treatments (p= 0.05) (Table 2.9). Callus induction medium supplemented with 2ml/l Previcur® had the lowest (0%) bacterial contamination while the highest bacterial contamination (16.7%) was in callus induction medium supplemented in 1 g/l Benlate (Table 2.9). ANOVA of explants contaminated with fungi also showed significant differences among the callus induction media (p= 0.05) (Table 2.9). The lowest fungal contamination (30%) was recorded in callus induction medium supplemented with 1 gl^{-1} Benlate while the highest (53.3%) fungal contamination was in callus induction medium supplemented with 2 mll^{-1} Previcur®. These results were again not satisfactory as they did not meet the required objective, although bacterial contamination was completely phased out in CIM supplemented with 1 gl^{-1} Benlate® (Table 2.9). However, this treatment was selected to be the best and further experiments aimed at optimizing that medium. The parameter to

investigate was the pH of washing treatments. The results showed that when explants were washed in liquid medium consisting of ¼MS, 5 gl^{-1} sucrose and 0.2 mgl^{-1} Previcur® at pH 8, there was no contamination by both bacteria and fungi recorded (Table 2.10). This leaf washing treatment was selected to be the most effective.

b) Effect of callus induction medium on callus production

Durand-Cresswell *et al.* (1982) reported the use of aseptically produced seedlings *in vitro* to circumvent the problems related to contamination of field-grown tissues. Also, Watt *et al.* (1991) and Hope (1993) reported the good response by young leaves in culture because they were free from contaminants. The complete loss of cultures due to microbial contamination when explants were taken from mature trees growing in the field, led into the establishment of eradication strategy discussed in section 2.3.2.a. Consequently, in this study, leaves of *E. saligna* (AS 196) obtained from *in vitro* grown shoots were used. They were cut and placed on four different callus induction media [Table 2.2 (section 2.2.4.b)] in the dark. After an incubation of four weeks, percentage explants with callus was recorded. The results indicated that no contamination occurred in any of four treatments and explants produced calli to almost the same extent (40- 47 %) (Table 2.11) (Plate.2.6). No further experimentation was carried out to regenerate plantlets from leaf discs because of time constraints.

Table 2.8: Effect of various treatments on eradication of endogenous contaminants from leaves of cold-tolerant *E. saligna* clone (AS 196). Leaf discs were plated on MS nutrients supplemented with 10 ml^{-1} 2,4-D, 30 gl^{-1} sucrose and 4 gl^{-1} Gelrite, pH 5.8 in Petri dishes. Explants were treated as depicted in Fig. 2.5. +w= washing overnight, -w= no wash, C1= 20 µl Rifampicin®+ 30 µl Trimethoprin® , C2= 100 µl Rifampicin® + 100 µl Streptomycin® and P= 2 ml^{-1} Previcur®. Levels of significant difference (ANOVA) are given with regard to the type of contaminant. Treatments with similar letters were not significantly different (P= 0.05).

	Explants with contaminantion	
Treatment	Bacteria (%)	Fungi (%)
wC1	8.2a	46.6a
wC2	16.7a	58.3ab
P+ C1+ wC1	35a	57.5ab
P+ C1+ wC1	22.4a	55.1ab
P+ C1- w	17.1a	48.8ab
P+ C2- w	37.5a	35b

Table 2.9: Effect of various treatments on eradication of endogenous contaminants from leaves of cold-tolerant *E. saligna* clone (AS 196). Explants were treated as depicted in Fig 2.6. Leaf discs were plated on medium described in Table 2.8. B= 1 gl^{-1} Benlate®, P= 2 mll^{-1} Previcur® and C2= 100 µl Rifampicin® + 100 µl Streptomycin®. Levels of significant difference (ANOVA) are given with regard to the type of contaminant. Treatments with similar letters were not significantly different (P= 0.05).

	Explants with contamination	
Treatment	**Bacteria (%)**	**Fungi (%)**
B	16.7a	30a
P	0	53.3ab
P+ B	6.7ab	40ab
P+ C2	4.2ab	45.8ab
P+ B+ C2	6.7b	43.3b

Table 2.10: Effect of pH of media used to wash leaves on the removal of endogenous contaminants. Explants were washed overnight in liquid medium consisting of ½MS nutrients and 5 gl^{-1} sucrose. B= 1 gl^{-1} Benlate®, P= 2 mll^{-1} Previcur® and C2= 100 µl Rifampicin® + 100 µl Streptomycin®. The CIM used consisted of MS nutrients supplemented with 1 mgl^{-1} 2,4-D, 1 gl^{-1} Benlate®, 30 gl^{-1} sucrose and 10 gl^{-1} agar, pH 5.8. Levels of significant difference (ANOVA) are given with regard to the type of contaminant. Treatments with similar letters were not significantly different (P= 0.05).

		Explant contamination (%)	
Wash Treatment	pH	Bacteria	Fungi
P	8	0	6.7a
B	6	0	6.7a
B	8	0	0

Table 2.11: Effect of concentration of 2,4-D on induction of calli for cold-tolerant *Eucalyptus saligna* clone AS 196. Callus induction medium (CIM) given in Table 2.1. Results were observed after 4 weeks of initiation. Levels of significant difference (ANOVA) are given with regard to the type of contaminant. Treatments with similar letters were not significantly different (P= 0.05).

Gelling agent (gl^{-1})	2,4-D (mgl^{-1})	Contamination (%)	Senescence (%)	Explant with callus (%)
Saarchem agar	1	0	33.3	46.7a
Saarchem agar	3	0	40	46.7a
Gelrite	1	0	60	46.7a
Gelrite	3	0	33.3	40a

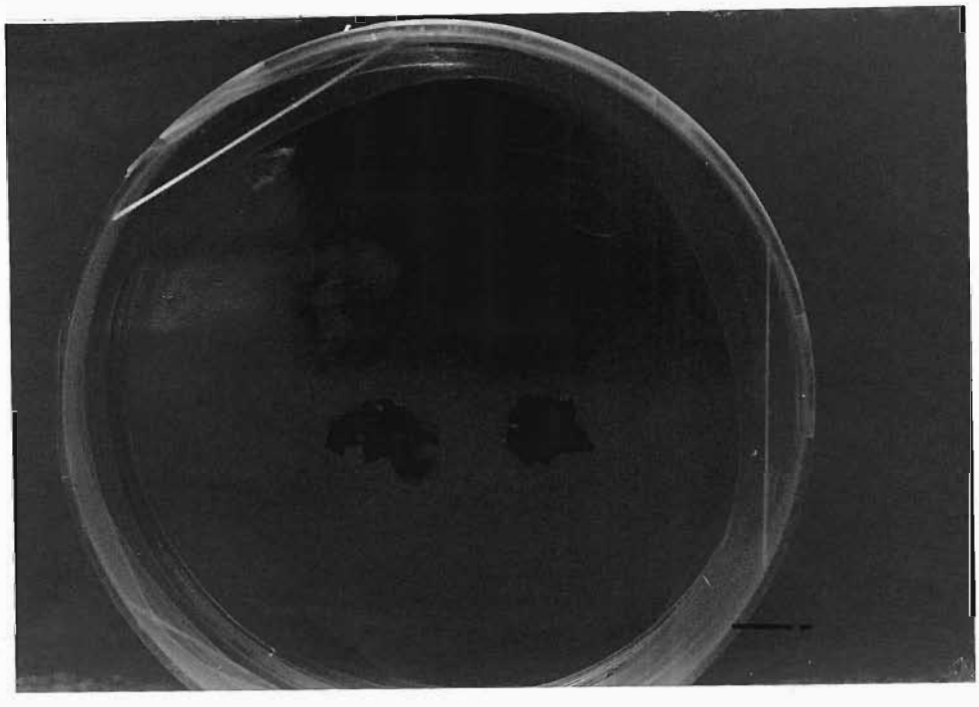


Plate 2.6: Callus produced from aseptic leaves derived from *in vitro* produced seedlings of *E. saligna* (AS 196) after 4 weeks in culture (bar = 3.5 mm).

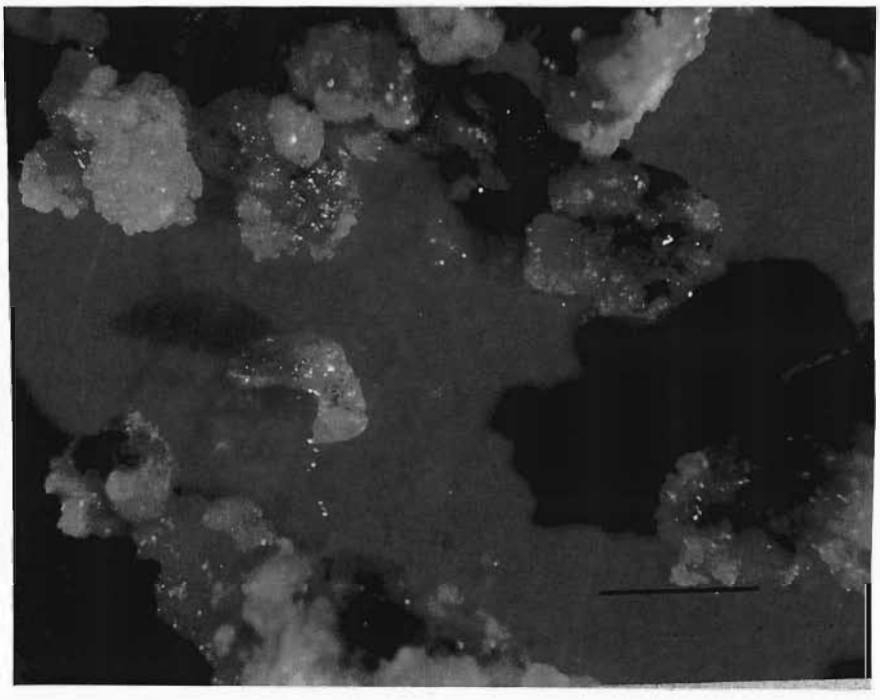


Plate 2.7: Callus mass derived from leaf explant of E. *saligna* (AS 196) (bar= 30 µm).

2.4 CONCLUSION

In the present investigation, a tissue culture method was developed to achieve relatively high frequency of bud induction, shoot multiplication and elongation from axillary buds of cold-tolerant clones *E. saligna* (AS 196), and *E. grandis* x *saligna* (PG 47 and PG 63). Plant clones derived from shoot apices and axillary buds often have been observed to be uniform, suggesting genetic stability. The optimised protocol for the production of buds from axillary buds involved inducing buds in induction medium comprising of MS supplemented with 20 gl^{-1} sucrose and 10 gl^{-1} Saarchem agar for 1-2 weeks. The induced buds were aseptically transferred to multiplication medium comprising of MS supplemented with 0.1 mgl^{-1} biotin, 0.1 mgl^{-1} calcium pantothenate, 0.2 mgl^{-1}BAP, 20 gl^{-1} sucrose and 3.5 gl^{-1} Gelrite for 4 weeks after which they were further transferred to elongation medium for 4-6 weeks comprising of MS medium supplemented with 0.1 mgl^{-1} biotin, 0.1 mgl^{-1} calcium pantothenate, 0.35 mgl^{-1} NAA, 0.1 mgl^{-1} kinetin, 0.1 mgl^{-1} IBA, 20 gl^{-1} sucrose and 3.5 gl^{-1} Gelrite. The results of this study showed that cytokinin (BAP) was a major limiting factor which controlled shoot multiplication *in vitro*, whereas auxin (NAA) had a limited promoting effect on shoot development.

Rooting in cold-tolerant *Eucalyptus* species remained a major problem. The presence of a brown exudate arising from the cut tissue lead to the subsequent death of the cultures. The amount of the exudate seemed to increase with age of the culture, but this was overcome by first storing/maintaining cultures in the dark for a period of at least seven days. The possibility that inhibitory factors are removed by repeated subculture also requires a rigorous investigation. Paton *et al.*, (1970) have developed a bioassay for root

inhibitory substances in *E. grandis* stems by using water in which stems are soaked. Future work should also consider investigations on this aspect with cold-tolerant clones of interest. *Ex vitro* rooting of *in vitro* derived shoots will probably have the potential as a labour saving step for large scale operations when high rooting ability is achieved.

The method for the propagation of cold-tolerant *Eucalyptus* species using leaf tissues from mature trees needs to be improved. The literature on somatic embryogenesis from mature leaf tissues is extensive but the methods are not effective with tissues of cold-tolerant *Eucalyptus* species. The requirement for obtaining suitable material for callus production was the use of young leaves from *in vitro* produced shoots.

CHAPTER 3

DEVELOPMENT OF PROTOCOLS FOR THE DIAGNOSIS OF COLD TOLERANCE PROTEINS.

3.1 INTRODUCTORY REMARKS AND REVIEW OF LITERATURE

Many years after its first use (Laemmli, 1970), polyacrylamide gel electrophoresis continues to play a major role in the experimental analysis of proteins and protein mixtures (Hames, 1990). Although two-dimensional gel separations of proteins have the highest resolving power, one dimensional polyacrylamide gel electrophoresis (1-D) is still the most preferred form of technique for many studies. This is because it offers sufficient resolution for most situations coupled with ease and the ability to process many samples simultaneously for comparative purposes (Hanash, 1985; Meza-Basso *et al.*, 1986; Rosas *et al.*, 1986; Cloutier, 1987; Mauk *et* al., 1989; Witt *et al.*, 1989; Gatschet *et al.*, 1994; Muthalif and Rowland, 1994; Ekramoddoullah *et al.*, 1995). The basic protocols for preparing and running one-dimensional polyacrylamide gels have changed relatively little in recent years but there have been considerable advances in the analysis of proteins separated by this method; for example silver staining (Morrisey, 1981; Merill and Goldman, 1984; Guy *et* al., 1988; Guy and Huskell, 1988; Robertson *et al.*, 1995;) and an immunoblotting methodology (Cooper and Ort, 1988; Ghosh *et* al., 1989 ; Hon *et* al., 1994; Boothe *et al.*, 1995).

3.1.1 Electrophoresis

a) Definition

Electrophoresis is defined as the transport of electrically charged particles in liquid media under the influence of a direct current electrical field (Thormann and Mosher, 1985), -as charged ions or groups migrate when placed in an electric field. The foundations of electrophoresis were laid last century when experiments were reported showing the effect of electric fields on charged particles, including proteins (Pornet, 1816; Quincke, 1861; Hardy, 1899). Since proteins carry a net charge at any pH other than their isoelectric point (equal number of positive and negative charges), they too will migrate and their rate of migration will depend upon the charge density (the ratio of charge to mass) of proteins concerned. The higher the ratio of charge to mass the faster the molecule will migrate (Morris, 1974; Schultz and Schimer, 1979). The application of an electric field to a protein mixture in a solution will therefore result in different proteins migrating at different rates towards one of the electrodes. In sodium dodecyl sulfate (SDS), migration is determined not by intrinsic electrical charge of polypeptides but by molecular weight.

b) Zone electrophoresis

As early as the nineteenth century British workers who measured mobilities of inorganic ions used gels as anti-convective media. For proteins, gels were first used in 1946, by British workers (Consden *et al.*, 1946). Another worker, Raymond (1959), introduced starch gel as an electrophoresis medium with a spectacular gain in resolution: 20-25 serum components could be recognised as compared to 5-6 components by convectional

methods. The improved resolution was intepreted as molecular sieving, a principle that a few years later gained immense importance through the introduction of the synthetic polyacrylamide gel by Raymond (1959) and collaborators. The success of gel electrophoresis is highly dependent on the quality of the starch gel itself. Being prepared from a biological product , it is not reproducibly good and may contain contaminants which can adversely affect the quality of the results obtained (Brewer, 1970; Hames, 1981; 1990).

A few years later, Ornstein (1964) and Davis (1964) devised a quite specific method of using the new gel, one goes under the name of disc electrophoresis, meaning that the very narrow proteins obtained in cylindrical gels appeared disc-like. Zone electrophoresis (ZE) is a modification of this procedure whereby the mixture of molecules to be separated is placed as a narrow zone or band at a suitable distance from the electrodes such that, during electrophoresis, proteins of different mobilities travel as discrete zones which gradually separate from each other as electrophoresis proceeds. On the other hand, polyacrylamide gel, as a synthetic polymer of acrylamide monomer, can always be prepared from highly purified reagents in a reproducible manner provided that the polymerization conditions are standardised. Polyacrylamide gel has the advantages of being chemically inert, stable over a wide range of pH, temperature, and ionic strength, and is transparent (Nielsen, 1985). Finally, polyacrylamide gel is better suited to a size fractionation of proteins, since gels with a wide range of pore sizes obtainable with starch gels is strictly limited.

However, according to Hames (1990), there are different disadvantages to zone electrophoresis in free solution. First, any heating effects caused by electrophoresis can result in convective disturbance of the liquid column and disruption of the separating protein zones. Second, the effect of diffusion is constantly to broaden the protein zones and this continues after electrophoresis has been terminated. To minimise these effects, zone electrophoresis of proteins is rarely carried out in free solution but instead is performed in a solution stabilised within a supporting medium. As well as reducing the deleterious effects of convection and diffusion during electrophoresis, the supporting medium allows the investigator to fix the separated proteins at their final positions immediately after electrophoresis and thus avoid the loss of resolution which results from post-electrophoretic diffusion. The fixation process employed varies with the supporting medium chosen (Hames, 1990).

c) Evaluation of pore size

The determination of pore sizes was reviewed by Rodbard (1976) and Stellwagen (1987). Pore sizes in polyacrylacrylamide gel may be pre-determined by either of the following two ways (Hoefer, 1994). One way is to adjust the total percentage of acrylamide, that is the sum of weights of the acrylamide monomer and the cross-linker (Chrambach, 1983). This is expressed as %T. As the %T increases, the pore size decreases. The other way to adjust pore size is to vary the amount of crosslinker expressed as a percent of the sum of monomer and crosslinker or %C (Rodbard *et al.*, 1976; Griffiths, 1979). When there is a wide range in the molecular weights of the material under study, the researcher may

prepare a pore gradient gel. The pore size in a gradient gel is larger at the top of the gel than at the bottom; the gel becomes more restrictive as the run progresses. By forcing the protein sample to migrate, in the direction of decreasing porosity, each protein will be stopped at a position where the pores are too small for further penetration (Rilbe, 1983). In this way, an elegant method for protein analysis according to molecular size was devised.

3.1.2 Properties of polyacrylamide gel

a) Chemical structure

Polyacrylamide gel results from the polymerisation of acrylamide monomer into long chains and crosslinking of these by bifunctional compounds such as N,N′-methylene bisacrylamide (usually abbreviated to bisacrylamide) (Fig. 3.1) reacting with free functional groups at chain termini (Schuler and Zielinski, 1988; Hames, 1981, 1990; Hoefer, 1993, 1994). Other crosslinking reagents have also been used to impart particular solubilization characteristics to the gel for special purposes.

b) Polymerization catalysts

Polymerization of acrylamide is initiated by the addition of either ammonium persulphate or riboflavin (Marshall and Inglis, 1985). N,N,N′,N′-tetramethylethylene-diamine (TEMED) accelerates the polymerization of acrylamide and bisacrylamide by catalysing the formation of free radicals from ammonium persulphate. Ammonium persulphate decomposes slowly, and fresh solutions should be prepared weekly.

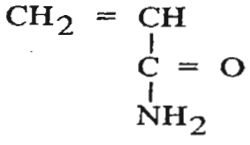
Since the free base of TEMED is required, polymerisation may be delayed or even prevented at low pH. The volumes of ammonium persulphate solution and TEMED cannot be generalized but refer to particular polymerization conditions (Görg *et al.*, 1988). Increases in either the TEMED or ammonium persulphate concentration increase the rate of polymerisation. In contrast to chemical polymerisation with persulphate, the use of the riboflavin TEMED system requires light to initiate polymerisation (Chrambach, 1985).

This causes photo-decomposition of riboflavin and production of the necessary free radicals. Although gelation occurs when the solutions containing only acrylamide and riboflavin are irradiated, TEMED is usually also included since under certain conditions polymerization occurs more reliably in its presence. Oxygen inhibits polymerization and so gel mixtures are usually degassed prior to use (Schuler and Zielinski, 1988).

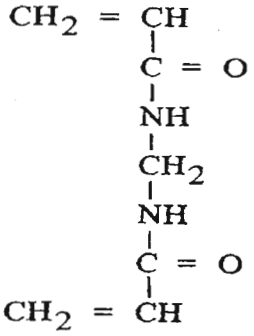
3.1.3. Experimental approach

a) Extraction of plant proteins and solubilization

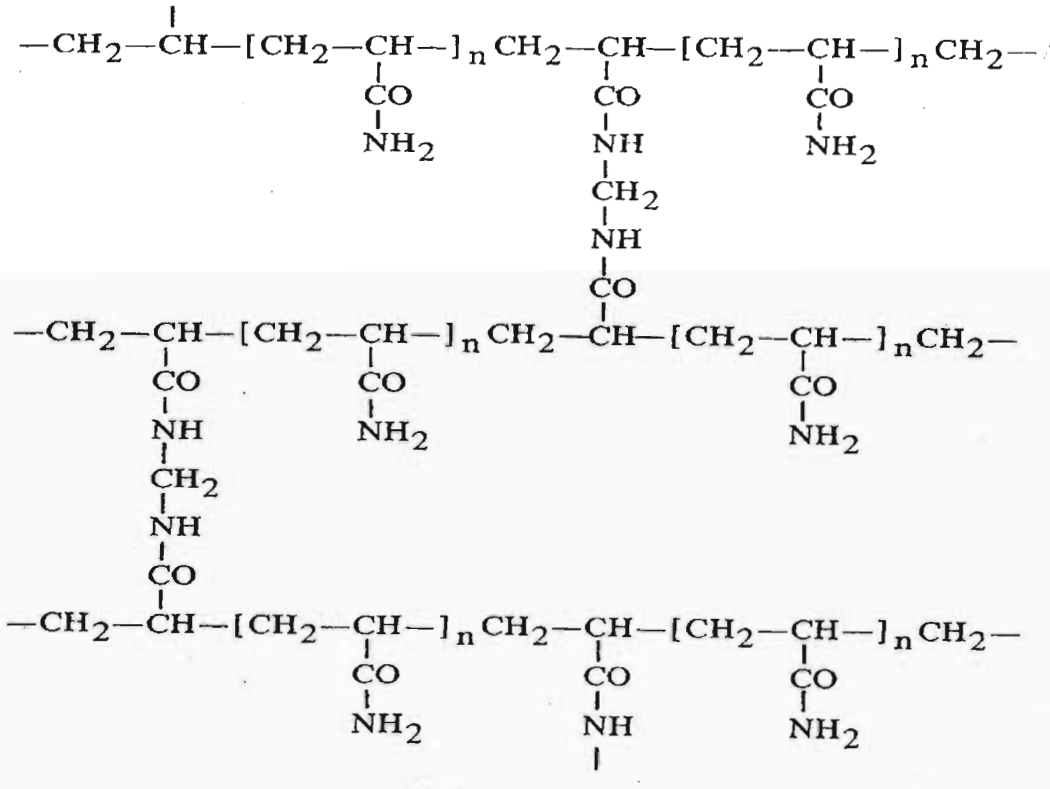
Soluble proteins can easily be extracted, but the solubilization of membrane proteins is more difficult and may require different additives (Damerval *et al.*, 1985). Generally proteins are solubilized, then precipitated if a higher concentration is necessary, and then resolubilised. Sometimes, the first solubilization step is omitted, and the proteins are directly precipitated. Occasionally, only one solubilization step is employed. Different



Acrylamide



N,N'—methylene bisacrylamide



Polyacrylamide gel

Fig. 3.2. The chemical structure of acrylamide, N,N'-methylene bisacrylamide, and polyacrylamide gel. (Reproduced from Hames and Rickwood, 1990).

extraction procedures are available, some of them are summarised in Table 3.1. The extraction of soluble proteins in a native state is performed in the presence of a buffer in order to counteract the reduction in pH which would follow rupturing of the vacuoles. The commonly used buffer is Tris-HCl (Damerval *et al.*, 1985).

Total proteins are usually obtained by extraction under denaturing conditions. In addition to soluble proteins, membrane bound proteins can also be extracted in a soluble form by boiling in the presence of sodium dodecyl sulphate (SDS) (Harrison and Black, 1982). This compound may also be added to a Tris buffer (Laemmli, 1970). Tris buffer is an ionic detergent, one of the best denaturing agents and denaturation is usually irrervesible. It destroys tertiary and quaternary structures, unfolds proteins, and destroys the majority of protein intractions (Gallagher and Leornard, 1987). Moreover, it is an excellent solubilizing agent. Care must be taken to avoid alterations induced by overheating such as aggregation of high-molecular weight polypeptides, observed for the catalytic subunit of ATPase (Gallagher and Leornard, 1987) or loss of resolution. Sodium dodecyl sulphate has to be pure in order to avoid streaking. Leaving out the first solubilization step, total proteins can be directly precipitated with trichloroacetic acid (TCA) (Meyer *et al.*, 1984). Trichloroacetic acid extraction is followed by washing with acetone to remove TCA (and chlorophyll) from the precipitate. In all these procedures, cellular fragments and proteins are separated by centrifugation after resolubilization of total proteins.

b) Type of organ/tissue for protein extraction

The choice of organ or tissue type has been equally varied for the purposes of protein extraction. Organs including roots (Dhugga *et al.*, 1988; Hilbert *et al.*, 1991; Hurkman *et al.*, 1991; Ni and Beevers, 1991; Breviario *et al.*, 1992), stems (Taylor *et al.*, 1991; Tseng and Li, 1991), leaves (Cooper and Ort, 1988; Boothe *et al.*, 1995), bark (Wetzel *et al.*, 1989), fruit, shoots (Cloutier, 1987), seedlings (Meza-Basso *et al.*, 1986; Mohapatra *et al.*, 1987), and cells (Robertson *et al.*, 1995) have been for protein extraction. *In vitro* cultures, derived from various explant-types, have also been used (Bon and Monteuuis, 1991; Ramagopal and Carr, 1991; Reinboothe *et al.*, 1992).

c) Problems raised by interfering compounds

The preparation of protein samples for electrophoresis is complicated by the interaction between proteins and other compounds, which are compartmented *in vivo* but which are mixed during grinding: proteases, phenolic compounds, terpenoids, pigments, lipids, inhibitory ions *etc.* (Damerval *et al.*, 1985). For example, seeds contain a lot of proteases, leaves a lot of pigments and phenolic compounds. Plant cells usually have large vacuoles which contain great amounts of proteolytic enzymes, accounting for 70 to 80% of the material present in the protein degradation pathway. The role of proteases is to regulate metabolic pathways, to eliminate abnormal proteins and to mature polypeptides (Meyer *et al.*, 1984). Those workers also compared protein patterns obtained with and without inhibitors and noticed no effect, but endogenous proteolytic activity in protoplasts of tobacco mesophyll seemed to be low. Proteins that are susceptible to breakdown by

proteases released from organelles are protected from being denatured when working with subcellular fractions.

When proteins are extracted with buffer, usually Tris-HCl, protease activity is maintained (Damerval *et al.*, 1985). Different protease inhibitors may be added in order to limit proteolytic degradation. The most commonly used inhibitor is phenylmethylsulphonyl fluoride (PMSF), an inhibitor of serine proteinases (e.g. trypsin), usually at a concentration of 1 mM. This may be used alone (Potter and Black, 1982; Tymms *et* al., 1982; Meza-Basso *et* al., 1986; Uemura and Yoshida, 1986) or in combination with leupeptin (Dasgupta and Bewley, 1984), a thiol proteinase inhibitor, also inhibiting several serine proteinases, or with SDS (Vierling and Key, 1984).

Harrison and Black (1982) have developed a protein extraction procedure in the presence of high amounts of SDS and 2-mercaptoethanol. The mixture is heated to 100 °C for 3 min. This treatment is sufficient enough to stop enzyme activity thereby halting the effect of proteases through denaturation. Simply grinding with SDS, without boiling, does not always prevent protein degradation since some proteolytic enzymes are active in solutions containing SDS prior to boiling (Vierling and Key, 1985). Homogenization of plant tissues in 10% (w/v) TCA sometimes is sufficient to inactivate the proteases which do not resume their catalytic function when resuspended in a buffer (Damerval *et al.*, 1986). Extraction with a mixture of TCA and acetone also avoids proteolysis.

Most plant tissues contain a large variety of phenolic compounds. These are accumulated in the vacuoles in various soluble forms; e.g. flavonoids and tanins (polymeric compounds of M_r between 300 and 5000, capable of precipitating proteins) (Harrison and Black, 1982). This paper also highlighted that working with untreated green plant tissues without removal of phenolic compounds (and pigments) leads to irreproducible results due to charge heterogeneity and streaking. Terpernoids, pigments and lipids can be removed during acetone precipitation since they are acetone soluble (Harrison and Black, 1982).

When proteins are extracted with aqueous buffers, oxidases are active and for this reason, complexing agents are generally not used alone, but in combination with reducing agents or antioxidants which prevent oxidase activity (Chory *et al.*, 1987). Ascorbate is commonly used as a reducing agent in concentrations varying from 1 mM to 250 mM. Similarly, antioxidants are also available including: 2.5 mM potassium metabisulphite (Uemura and Yoshida, 1984), 1-100 mM dithiothreitol (DTT), thioglycolate, cysteine, 40 mM 2-mercaptoethanol (Tymms *et al.*, 1982) and 10 mM diethyl-dithiocarbamate (DIECA). Some ways of limiting proteolysis during extraction are summarized in Table 3.2.

3.1.4 Some of the staining procedures for proteins

Protein bands of sufficiently high concentration may be localised by direct photometric scanning of unstained gels at 280 nm (Hames, 1990). The gel is placed in a suitable

quartz trough and scanned using suitable modern spectrophotometer fitted with a gel scanning attachment. Various negative stains have been reported but for quantitation purposes protocols which give positively stained protein bands against a pale background are preferred (Cloutier, 1987; Cooper and Ort, 1988).

a) Coomassie Blue staining

After electrophoresis, the proteins are usually located by staining with Coomassie Brilliant Blue (CBB). The staining solutions contains 0.15% (w/v) CBB G250 in methanol/glacial acetic acid/water (50:10:40, by vol.) (Marshall and Inglis, 1986). The CBB is dissolved in methanol before adding acetic acid and water and the solution is filtered. Destaining is conventionally carried out overnight in a solution containing methanol/glacial acetic acid/water (5:7.7:87.5, by vol.). Most of the early work used Amido Black 10B as a general protein stain but this has been largely superseded by Coomassie Blue R-250 (Reddish hue) which is more sensitive (Schuler and Zielinski, 1988). Another protein stain with almost the same sensitivity as Coomassie Blue R-250 is Xylene Cyanine Brilliant G (Coomassie Blue G-250; G = greenish hue). Coomassie Brilliant Blue R-250 was used to stain gels at 0.1% in methanol: water: acetic acid (Cloutier, 1987, Schuler and Zielinski, 1988; Arora, 1995).

b) Silver staining

Silver staining was introduced in 1979 by Switzer *et al.* as a novel staining procedure up to 100 times more sensitive than Coomassie Blue R-250, able to detect as 0.38 ng/mm^2 of

Table 3.1: A list of some of the procedures used in extracting proteins. P= membrane proteins re-extracted from the pellet after native extraction of soluble proteins. S= soluble; MB= membrane-bound; T= total proteins.

Nondenaturing extraction	Denaturing extraction	Precipitation	Protein class	Material	References
Tris		Acetone	S	Rape seedlings	Meza-Basso (1986)
Carrier ampholytes		-	S	Various leaves, roots...	Mayer *et al* (1987)
Tris	SDS (P)	Ammonium sulfate	S+MB	Crab grass leaves	Potter and Black (1982)
Tris	SDS(P)(hot)	-	S+MB	Broad bean root cells	Theillet *et al* (1982)
Tris	Lysis buffer (P)	Acetone	S+MB	Maize and pea shoots	Chory *et al* (1987)
	SDS(hot)	Acetone	T	Crab grass mesophyll and bundle sheath cells	Harrison and Black (1982)
	SDS(hot)	Acetone	T	Wheat leaves	Colas des Francs *et al* (1985)
Tris	Tris+SDS(hot)	-	MB	Winter rye seedlings	Uemura and Yoshida (1984)
		Acetone	T	Soybean cell cultures	Vierling and Key (1985)
	Phenol	Ammonium acetate	MB	Microsomal suspension from barley roots	Hurkman and Tanaka (1986)
	Tris+SDS(hot)	-	T	Pea seedlings	De Vries *et al* (1982)

Table 3.2. Additives used to limit the proteolysis during native extraction.

Native extraction in the presence additives (TCA, SDS)	References
50 μgmL^{-1} PMSF	Meza-Basso *et al* (1986)
2 mM PMSF	Hurkman and Tanaka (1986)
1 mM PMSF	Uemura and Yoshida (1984)
	Vierling and Key (1985)
1 mM Chymostatin, 1 mM elastatinal, 1 mM pepstatin	Colas des Fransc *et al* (1985)
1 mM Antipain, 1 mM leupeptin, 1 mM pepstanin	Colas des Fransc *et al* (1985)
8 mM NEM, 1 mM TPCK, 5 mM TLCK	Theillet *et al* (1982)

bovine serum albumin. A more sensitive method involves equilibration of the gel with silver nitrate solution, then treatment with a reducing solution followed by equilibration in a solution containing sodium carbonate which enhances the appearance of the polypeptide-silver complexes (Morrisey, 1981; Merill and Goldman, 1984; Guy *et* al., 1988; Guy and Huskell, 1988; Robertson *et al.*, 1995). It may be used after the Coomassie Blue procedure with a large increase in sensitivity if the dye is carefully washed out with methanol/acetic acid. Prior to silver staining, the proteins need to be fixed. This fixation step has two roles to play, *viz.* to immobilize the proteins in the gel, or at least greatly retard their diffusion from the gel and secondly, it removes substances which may interfere with the staining procedures, such as detergent, reducing agent or reactive buffer components like glycine.

A variety of fixatives for silver staining have been used, including glutaraldehyde (Oakley *et* al., 1980), ethanol or methanol and acetic acid (Morrisey, 1981), and TCA (Confavreux *et* al., 1982). A key factor for success in silver staining after SDS-PAGE is the removal of SDS prior to staining. Because the method is sensitive, scrupulous cleanliness is required in the preparation of the glass plates (Marshall and Inglis, 1986). Tracking of the samples obtainable between the plate and the gel does not adhere to the glass.

3.1.5 Cold tolerance proteins

a) Background

Low, non-freezing temperatures induce genetic and metabolic changes, enabling plants to develop cold hardiness (Guy, 1990; Thomashow, 1990). During this cold-acclimation process a general increase in soluble proteins has been observed (Guy *et al.*, 1985; Meza-Basso *et al.*, 1986; Johnson-Flanagan and Singh, 1987; Mohapatra *et al.*, 1987; Cattivelli and Bartels, 1989; Lee *et al.*, 1991). This increased protein content is associated with qualitative alteration in the population of translatable mRNAs during cold-acclimation in many plant species. For example, an alteration in proportion or size of polysomes is indicative of metabolic change. This association has been documented in seed development (Gwo'z'dz' and Deckert, 1989), tissue differentiation (Mösinger and Schopfer, 1983) and in response to environmental stresses (Fehling and Weidner, 1986; Berry *et al.*, 1989). The polysome profiles have been used as an indicator of protein synthesis activity after low temperature treatments (Fehling and Weidner, 1986; Laroche and Hopkins, 1987; Bocharova and Klyachko, 1988; Perras and Sarhan, 1990).

b) Detection of cold tolerance proteins in plants

Prior to the advances in understanding anaerobic and heat shock responses, there was considerable evidence to suggest that cold tolerant or cold acclimated plants contained unique proteins (Rochat, 1975; Levitt, 1980). Weiser, as early as 1972, proposed that altered gene expression and the synthesis of new proteins during cold acclimation was necessary for the induction of greater freezing tolerance in temperate perennials. The first direct evidence to support an alteration in gene expression associated with the cold acclimation process was the observation that newly translatable mRNAs were induced in

spinach leaf tissues exposed to 5 °C (Guy *et al.*, 1985). The synthesis of three of these high molecular weight cold acclimation polypeptides in leaf tissue (CAPs) Mr 160 000, 117 000 and 85 000, during cold acclimation was highly correlated with the induction of freezing tolerance.

A positive correlation of the expression of cold-regulated genes with freezing tolerance has been observed in several plants (Mohapatra *et al.*, 1989; Binh and Oono, 1992). Consistent with these findings, changes in protein synthesis also have been positively correlated with frost hardiness (Pomeroy and Siminovitch, 1970; Kang and Titus, 1980). Cold acclimation was closely paralleled by an accumulation of membrane-bound proteins in the leaves of boxwood (*Boxus microphylla* var. *koreana* Nakai; Gusta and Wesser, 1972). Crude protein extracts obtained from cold-acclimated leaves of spinach (*Spinach oleracea* L.) and some galactose-specific lectins were shown to protect isolated thylakoid membranes against freeze-thaw damage (Hincha *et al.*, 1989; 1990; 1993). Removal of apoplastic proteins that were accumulated during cold acclimation of leaves of winter rye (*Secale cereale* L.) increased the level of injury of these leaves caused by freezing. These workers suggested that the apoplastic proteins might have ice-nucleating and anti-freeze properties of controlling extracellular ice formation in leaf tissues, a mechanism thought to operate in insects and marine invertebrates (Storey and Storey, 1988).

Some polypeptides encoded by cold-regulated genes are of low molecular mass (Johnson-Flanagan and Singh, 1987) and have the unusual property of remaining soluble upon

boiling (Lin *et al.*, 1990; Lin and Thomashow, 1992). An increase in soluble proteins was found to be associated with frost hardiness in *Pinus* species (Pomeroy and Siminovitch, 1970). A 19-kD protein designated as *Pin l* I was found to accumulate in large amounts in samples collected as fall progressed and could not be detected in samples collected the following summer.

c) Recent reports on cold tolerant proteins in plants

There is evidence from studies with numerous herbaceous plant species, *Arabidopsis thaliana* (Kurkela and Franck, 1990; Nordin *et al.*, 1991; Gilmour *et al.*, 1992), alfalfa (Mohapatra *et al.*, 1989), *Brassica* (Meza-Basso *et al.*, 1986) that low, non-freezing temperatures induce the accumulation of specific proteins in leaves and stems. The question of how low-temperature exposure or chilling units (CU) accumulation is involved in the development of cold hardiness in woody perennials is poorly understood. A group of proteins called BSPs has been reported in apple (O'Kennedy and Titus, 1979), elder (Nsimbu-Lubaki and Peumans, 1986), maple, willow, poplar (Wetzel *et al.*, 1989; Coleman *et al.*, 1991), and peach (Arora *et al.*, 1992). These have been shown to accumulate in protein storage vacuoles of inner bark parenchyma cells during autumn and winter (Greenwood *et al.*, 1986; 1990; Sauter *et al.*, 1988; Wetzel *et al.*, 1989). The function of BSPs is unknown but it has been suggested that they might play a role in dormancy development, cold acclimation and/or N_2 storage (Guy, 1990; Coleman *et al.*, 1991; Arora *et al.*, 1992). In another study (Muthalif and Rowland, 1994), blueberry plants (*Vaccinium*, section *Cyanococcus*) were used to investigate changes in gene

expression in floral buds of woody perennial in response to CU accumulation and development of cold hardiness. The results of that study showed accumulation of CU associated with an increase in the level of several peptides. The levels of three polypeptides of 65, 60 and 14 kD increase most dramatically with CU accumulation.

d) Other stress related proteins

Recently, it has become apparent that plants respond to adverse environmental stress conditions through alterations in protein synthesis patterns (Manson *et al.*, 1988). In this regard, when the environmental conditions become unsuitable for optimal growth and development of the plant, a set of new stress related proteins are synthesized. The newly synthesized proteins appear to be more or less specific to a given environmental stress condition, i.e. anaerobic proteins will be synthesized for anoxia and anaerobiosis, heat-shock proteins for supra-optimal temperatures, and water stress proteins for water deficits (Heikkila *et al.*, 1984). Above a threshold temperature, the normal pattern of protein synthesis is repressed and a new set of heat-shock proteins (HSPs) is synthesized from newly translated mRNA. There are a number of major families of HSPs, classified on the basis of molecular weight; high-molecular-weight (approximately 70 kD) and low-molecular-weight (approximately 14-30 kD). The heat-shock response is not unique to plants but is found in every organism so far examined (Schlesinger *et al.*, 1982; Nagao *et al.*, 1990). For example, the temperature at which HSPs accumulate maximally is 45 °C in 1-d-old pearl millet and sorghum seedlings (Howarth, 1989), 40 °C in soybean and

maize (Key *et al.*, 1981; Cooper and Ho, 1983), and 37 °C in *Brassica oleracea* (Fabijanski *et al.*, 1987).

Other examples include synthesis of starvation proteins in parsley cell cultures (Walter and Hahlbrock, 1985), wound-induced proteins in maize (Bonham-Smith *et al.*, 1988), or proteins of pathogenesis in pea and parsley (Daniels *et al.*, 1987).

3.1.6 Aims of this investigation

It has become apparent from many published findings that plants respond to adverse environmental stress conditions through alterations in protein synthesis patterns. This allows the plant to synthesise a set of new stress related proteins when the environmental conditions become unsuitable for optimal growth and development of the plant. Although the functional identity of most of these stress-induced proteins remains undetermined, it seems quite certain that some of them will function to enhance plant survival. In this study situation, it also hypothesised that the same happens cold-tolerant *Eucalyptus* clones, that a polypeptides responsible for cold-tolerance might be produced in response to low temperatures. However, a review of the literature indicated that an electrophoresis method, specific for leaf proteins of *Eucalyptus*, is not available. Consequently, the aims of this investigation were:-

1. To establish methodology for 1D-gel electrophoresis of *Eucalyptus* leaf protein.
2. To investigate the presence of cold-induced polypeptides in response to cold treatment in *Eucalyptus* for future use as a diagnostic test for identification of cold-tolerant clones.

3.2 MATERIALS AND METHODS

3.2.1 Plant material

Leaves of the non-cold tolerant clone *E. grandis* (TAG 731) and of the cold-tolerant clones *E. saligna* (AS 196) and *E. saligna* (AS 184), selected from Australia, *E. saligna* (TS 15), selected in Transvaal, and *E. grandis* x *nitens* (GN 1) were used. All these clones were supplied by Mondi Forests (Hilton, SA). The plants were grown in soil under greenhouse conditions 25 °C/20 °C day/night cycle with relative humidity of 80 %.

3.2.2 Short-term cold treatment

Chilling was carried out for 12h at 4 °C in the dark cold room. Non-chilled control plants were maintained in the greenhouse.

3.2.3 Reagents

Coomassie Brilliant Blue, 2-mercaptoethanol, phenylmethylsulphonyl (PMSF) were obtained from Sigma. Triton X-100 was obtained from Schwartz/Mann. Other reagents included acrylamide, ammonium persulphate, methylenebisacrylamide, sodium dodecyl sulphate (SDS), glycine, tetramethylenediamine (TEMED), glycerol, dimethylsulphoxide (DMSO) (all from Merck, SA) and molecular weight markers (14 - 340 kD) (Combithek®, Boehringer Mannheim, GmbH). All reagents used were of analytical grade or the best grade available.

3.2.4 Colorimetric assay for protein determination

a) Preparation of the protein reagent

The reagent was prepared according to Bradford (1976). Coomassie Brilliant Blue G (0.05 g) was dissolved completely (6 hours) in 25 ml of 95% (v/v) ethanol. To this solution, 50 ml of 85 % (v/v) orthophosphoric acid was added, and made up to 500 ml with distilled water. Finally, the whole mixture was filtered in double Whatmann's No.1 filter papers and the filtrate was stored in a dark bottle at 4 °C in the fridge for two weeks only.

b) Protein determination

The method of Bradford (1976) was used. Protein solution containing 0 to 500 μg protein in a volume of 0.1 ml was pipetted into Eppendorf tubes. The volume in the Eppendorf tubes was adjusted to 1 ml with the extraction buffer (0.2 M Tris-HCl - pH 7.5, 1M sucrose and 0.056 M 2-mercaptoethanol). An amount of 0.1 ml of this solution from Eppendorfs was pipetted into five milliliters of protein reagent in glass test tubes (12 x 100 mm) and the contents were mixed gently. Samples were read at 595 nm against a reagent blank prepared from 0.1 ml of the extraction buffer and 5 ml of the protein reagent. The standard curve was prepared with bovine serum albumin (BSA) in extraction buffer (0.2 M Tris-HCl - pH 7.5, 1M sucrose and 0.056 M 2-mercaptoethanol) and a range of 0 - 50 μg/0.1 ml was used.

3.2.5 Protein extraction

Two methods were attempted (see below) and method two was adopted due to its better protein extraction potential (as discussed in the results section).

a) Method 1

An amount of 1.5 g fresh leaf material was weighed into a test tube containing 1.5 ml of the extraction buffer (0.2 M Tris-HCl - pH 7.5, 1M sucrose and 0.056 M 2-mercaptoethanol). The mixture was homogenized using a Ultra-Turrax tissue homegeniser (Janke and Kunkel, FRG) until it was finely mixed, after which another 1 ml extraction buffer was added. The sample was then centrifuged at 29,000 g for a period of one hour at 4 °C. The resulting supernatant was kept for assay and precipitation.

b) Method 2

Leaf material (0.5 g) was ground in liquid nitrogen with pestle and mortar, and 2.5 ml extraction buffer was added to the finely ground powder. The homogenate was centrifuged for one hour at 4 °C at 29,000 g. A sample of the supernatant was assayed colorimetrically for protein (Bradford, 1976) and the rest was used in the precipitation step.

3.2.6 Protein precipitation

Two protein precipitation methods were used with respect to the protein extraction methods described in section 3.2.4 above. Method 1 of protein precipitation followed method 1 of protein extraction (described in the section above), whilst protein method 2 followed method 2 protein extraction.

a) Method 1

Aliquots amounts of the assayed supernatant measuring 2 ml were decanted into large plastic centrifuge tubes (10 ml) to which 6 ml of ice cold deionised water, and 50 µl 2 % (w/v) sodium deoxycholate were added. After shaking well, the mixture was allowed to stand for 15 minutes after which, 2 ml of ice cold 24 % (w/v) trichloroacetic acid (TCA) were added. The mixture was again shaken well and centrifuged for 40 minutes at 8,500 g using a refrigerated bench-top centrifuge at 4 °C. The supernatant was poured off and the pellet was air dried. The pellet was resuspended in 0.15 ml extraction buffer (section 3.2.5) and the resuspension was assayed colourimetrically (section 3.2.4.). The rest of the pellets were kept for electrophoresis in Sterile Cryo-Tubes (Greiner) in liquid nitrogen. For gel electrophoresis, the pellets were recovered from the liquid nitrogen storage and resuspended in 0.1 ml sodium dodecyl sulphate (SDS) gel buffer for loading gel. The gel loading buffer was [1.8 x 10^{-5} M ethylene diaminetetraacetic acid (EDTA), 1.1 x 10^{-2} M Tris-HCl (pH 8.8), 3.6 x 10^{-3} M sucrose and 3.6 x 10^{-5} M Dithiothreitol (DTT)].

b) Method 2

To 1 ml aliquots of supernatant containing 100 µg protein, 250 µl 100% TCA were added in Eppendorf tubes. The mixture was shaken well and allowed to stand for 2 hours, after which it was centrifuged for 45 minutes at 4 °C and 12,000 g. The supernatant was discarded and the pellet was resuspended in 1 ml ice-cold 100% acetone and re-centrifuged for 40 minutes. Further washings with acetone (in preliminary work) were found not to be necessary as they did not improve protein

purity and gel resolution. After acetone washing, the pellets were either resolubilised in 1) a 1:1 (v/v) mixture of 0.24 M Tris-HCl (pH 7.5) containing 20% 2-mercaptoethanol and 100% (DMSO) for colourimetric protein determination (Bradford, 1976) of recovery, or 2) sample loading buffer (section 3.2.5) for gel electrophoresis. When necessary, protein pellets were stored in a freezer at -4 °C, as described in section 3.2.6.

3.2.7 Gel Electrophoresis

The SE 600, the basic Hoefer unit for vertical slab gel electrophoresis was used. It accommodated two gel sandwiches, 16 X 18 cm in size. The method below describes a protocol for protein gel electrophoresis in a 12.5% acrylamide gel with a 6% acrylamide stacking gel using the discontinuous buffer system originally described by Laemli (1970).

a) Preparation of the gel mould

Glass plates were selected and carefully cleaned with warm soapy water, rinsed with distilled water, and dried before use. Four 1.5-mm-thick spacers were also selected so that two spacers were placed on both sides of the sandwich. One clean plate was placed on a clean surface so that a long edge (180 mm) faced towards the assembler. The spacer strips were laid along the two short (160 mm) edges of the plate. The spacers were allowed to extend a little beyond the edge of the plate. The second glass plate was placed on top of the spacers.

A clamp was slid loosely onto each 160 mm side of the glass plate sandwich. The spacers were pushed in on the clamps so that they moved in flush with the edges of the glass plate. One screw was loosely tightened on each clamp so as to hold the assembly together. The sandwich was held upright with the bottom edge resting on a flat surface and pushed gently on the top edge of the glass plates and spacers to align them with the clamps. The sandwich was laid flat and all the screws on both clamps were thumb tightened. A little vaseline was smeared on the bottom edge of the sandwich. The casting stand was leveled by means of the four screws inserted from the underside of the base. Clean solid rubber gaskets were placed in both casting cradles and a glass sandwich was inserted into each casting cradle, with the screws facing away from the sandwich. At this stage, the assembly was ready to receive the acrylamide solution (see below).

b) Preparation of the 12.5% acrylamide separating gel

An amount of 40 ml of separating gel solution was mixed in a 50 ml side-arm flask by adding ice-cold 12.5% (w/v) acrylamide stock, 0.38 M Tris-HCl (pH 8.8), 1 % (w/v) SDS and 18.1 ml deisonised water. The side-arm flask was stoppered and a vacuum was applied for 10 minutes or until the bubbles were completely removed. The flask was swirled gently for few minutes after which 300 µl 10 % (w/v) freshly prepared ammonium persulphate and 20 µl tetramethylethylenediamine (TEMED) were added. The flask was again swirled carefully avoiding any bubble creation. The solution was poured into the gel sandwich (section 3.2.7a) using a syringe. The gel was overlaid with water-saturated 100 % (v/v) isobutanol using a Pasteur pipette. The gel was allowed to set overnight.

c) Preparation of the stacking gel

The isobutanol surface was poured off from the top of the set gel and the gel top was rinsed with deionised water. The stacking gel solution was prepared in a 50 ml side-arm flask by mixing 6 % (w/v) acrylamide, 0.12 M Tris-HCl (pH 6.8), 0.1 % (w/v) SDS and 5.5 ml deionised water adding to a total volume of 10.5 ml. All the solutions were ice-cold with the exception of SDS that was kept at room temperature. The solution was evacuated as for the stacking gel solution. An amount of 75 µl of freshly prepared 10 % (w/v) ammonium persulphate, 5 µl TEMED were also added. About 10 mm of the stacking gel solution was used to rinse the top of the separating gel. After a rinse, the sandwich was filled to the top with the stacking gel mixture. The comb was inserted into the gel, taking care that no air bubbles were trapped under the teeth. The comb extended into the gel far enough to give a stacking gel of about 1 cm. The gel was allowed to set for roughly 30 minutes.

d) Sample preparation for gel electrophoresis

Pellets (of known amount of protein) that resulted from the precipitation step (section 3.2.5b) were first air-dried and then 100 µl of the sample loading buffer was added to them. The composition of this buffer was : 0.12 M Tris-HCl, 4 % (w/v) SDS, 10 % (v/v) 2-mercaptoethanol, 20 % (v/v) glycerol and 2 mg/l bromophenol blue. Prior to loading, the samples in the loading buffer were boiled for 5 minutes after which they were rapidly cooled on ice. In order to prepare the molecular weights markers (Combithek®, Boeringer Mannheim, GmbH), 5 µl of each of the individually-supplied protein solutions (range 14- 340 kD) were mixed to give a total volume of 40 µl. To this mixture, 40 µl of 250 mM Tris-HCl (pH 6.8), 4 % (w/v) sodium

dodecyl sulphate (SDS), 20 % (v/v) glycerol, 0.002 % (w/v) bromophenol blue and 1 % (w/v) dithiothreitol (DTT) were added.

Samples containing 50 μg protein were subjected to the 12.5 % SDS-PAGE 1D-gel electrophoresis described in section 3.2.6. The gels were run for 3.5-4 hours. The initial voltage and current settings were 130-140 V and 40 mA (constant), respectively.

e) Gel staining and destaining

Individual gels were stained in 100 ml of 0.25 % (w/v) Coomassie blue R250, 50 % (v/v) methanol and 10 % (v/v) acetic acid for 4 h. They were destained in 10 % (v/v) methanol and 7 % (v/v) acetic acid for 16 h, during which time the solution was changed 8 times.

f) Gel storage and photography

The gels were stored in heat-sealed plastic bags in a fridge at 4 °C for several weeks. The gels were photographed over a light box, using a Nikon FM2 camera with 60 mm lens (f16 and shutter speed 30) and monochrome film.

g) Gel analysis

Polypeptide spots were traced by hand onto transparent film. These traces were used for comparison of electrophotograms. Densitometric scans of gels were also performed using the GS 300 Densitometer scan (Hoefer Scientific Instruments, USA).

With this densitometer, protein regions were traced through peaks and profiles obtained and were used for comparative assessments.

3.3 RESULTS

3.3.1 Optimisation of protein extraction and precipitation steps

Different cold-tolerant clones and different leaves (with regard to age and position) were used for preliminary experiments because of the low availability of material. Therefore, variations in protein extraction were encountered.

a) Method 1

i) Homogenisation

This method was fully described in section 3.2.3 of materials and methods. The Ulta-Turrax tissue homogeniser (Janke and Kunkel, FRG) was found not to be particularly effective in homogenising leaf tissue. The leaf tissue was not adequately ground because after centrifugation, some broad leaf pieces could still be found in the supernatant. Further, the recommended temperature for protein extraction is less than 4 °C (Witt *et al.*, 1989; Marentes *et al.*, 1993), which is difficult to maintain using electric homogeniser which generated heat (even when the tube with the material was placed on ice, as in the case here). Failure to comply with this temperature limit could result in many proteins being lost due to denaturing at unfavourable temperatures. This method therefore caused difficulty in ensuring consistency in homogenising leaf tissue, resulting in high experimental variability. Nevertheless, investigation (see below)

were undertaken using this homogenisation method as relatively high levels of protein were obtained, despite the problems.

ii) The effect of extraction buffer on total protein extraction

Three extraction buffers were investigated with regard to yield of extracted protein. That buffer comprising 0.2 M Tris-HCl (pH 8.5), 1 M sucrose and 0.056 M 2-mercaptoethanol was selected for future work as, although it did not significantly increase the amount of protein extracted, it appeared to give slightly better results than the other two (Table 3.3).

iii) The effect of buffer pH on total protein extraction

The pH of the extraction buffer selected in section 3.3.1a.ii above was varied from pH 7.5 to pH 10.5 and the effect of buffer pH on total protein extraction investigated (Fig. 3.2). Protein yield was found to increase to a maximum at pH 9.0 and then remain constant up to pH 10.5.

iv) Protein recovery after precipitation

The aim of this investigation was to test protein recovery with precipitation method 1 (section 3.2.4). Extractions were done from the same plant and protein was measured before (expected) and after (observed) TCA precipitation (Table 3.5). Different amounts were also precipitated to determine efficiency of recovery with different amounts of proteins loaded on TCA. The results,as depicted in Table 3.4, indicated that regardless of the amount of protein loaded for precipitation, 40 - 50 μg/0.2 ml protein was always recovered. On the basis of these results,

samples containing less than 40 μg/0.2 ml were precipitated to test recovery efficiency (Table 3.5). This was found to be relatively high (i.e. 80 - 95 %) but there was a high degree of variability. However, the major drawback with this method was that 40 μg is less than the amount required to load on a gel as most workers recommend 50 - 100 μg (Wetter and Dyck, 1983; Hoefer, 1992; 1993; Boothe *et al.*, 1995; Robertson *et al.*, 1995). That is, if this protocol was to be used, many samples had to be precipitated and pooled to get enough protein per sample to be loaded on a gel. However, this was found to be very laborious and time-consuming. Also, when this was attempted, inaccurate results were obtained; when 50, 75 and 100 μg were loaded onto gels, bands appeared to be very faint, a clear indication of low, inaccurate and inconsistent amount of protein loaded (Fig. 3.3). It appeared that protein was either lost and/or had been overestimated in the quantitative assay. For those reasons, another method was investigated.

Table 3.3: The effect of buffer composition on protein extraction from leaf material. All buffers comprise 0.2 M Tris-HCl (pH 8.5) and 0.056 M 2-mercaptoethanol. Data are presented as means ± SE, n=3.

Buffer	mg protein/ g f mass
1M sucrose	6.2 ± 1.2
0.01 M EDTA 0.001 M PMSF	5.25 ± 0.46
0.01 M EDTA 0.001 M PMSF 0.5% (w/v) PVP	5.11 ± 0.11

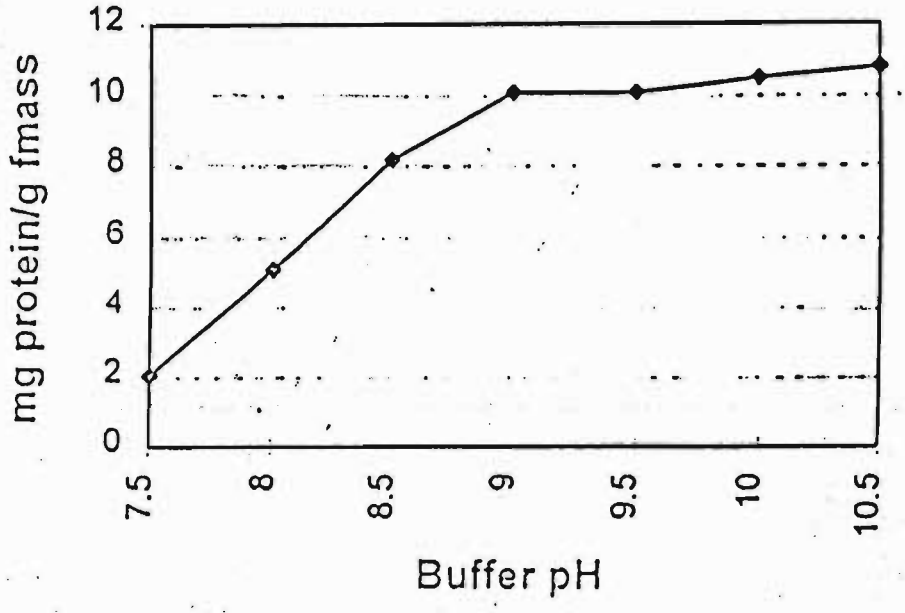


Figure 3.2: Effect of buffer pH on protein extraction from leaf material. Standard error smaller than symbols, n=3.

b) Method 2

i) Extraction of protein from leaf material

Of the two tested procedures, the most efficient one was found to be the following: after grinding 0.5 g fresh leaf material in liquid nitrogen with a mortar and pestle, 2.5 ml extraction buffer (0.2 M Tris-HCl (pH 7.5), 1 M sucrose and 0.056 M 2-mercaptoethanol) was mixed with the finely in the initial protein extraction procedure (section 3.2.3), some leaf pieces left in the supernatant were found to interfere with the colourimetric assay. The introduction of this step resulted in a lower yield than that obtained with method 1 for protein extraction, (using Ultra-Turrax tissue homogeniser) (Table 3.6), but avoided interference with colorimetric assay. As discussed previously (3.3.1), it appeared that method 1 of protein precipitation overestimated the percentage protein recovery, thus resulting in under loaded and therefore not well resolved gels.

Table 3.4: Effect of amounts of protein precipitated on subsequent recovery. An amount of 0.2 ml protein extract (supernatant) was precipitated with 6 ml dH_2O, 3 ml 24% (w/v) TCA and 50 µl 2% (w/v) sodium deoxycholate. n=3.

Sample	Amount of protein precipitated (µg)	Amount of protein observed (µg)	% Recovery
1	195	204 ± 34.8	100
2	288	216 ± 14.8	75
3	477	240.8 ± 30.6	50.5

Table 3.5: An investigation into the consistency of protein recovery when the sample for precipitation contained 40 µg/0.2. n=3 and data presented as means ± SE.

Sample	Observed µg/0.2 ml	% Protein recovery
1	32.3 ± 14.1	85
2	39.8 ± 5.1	94.8
3	37.2 ± 12.4	80.2

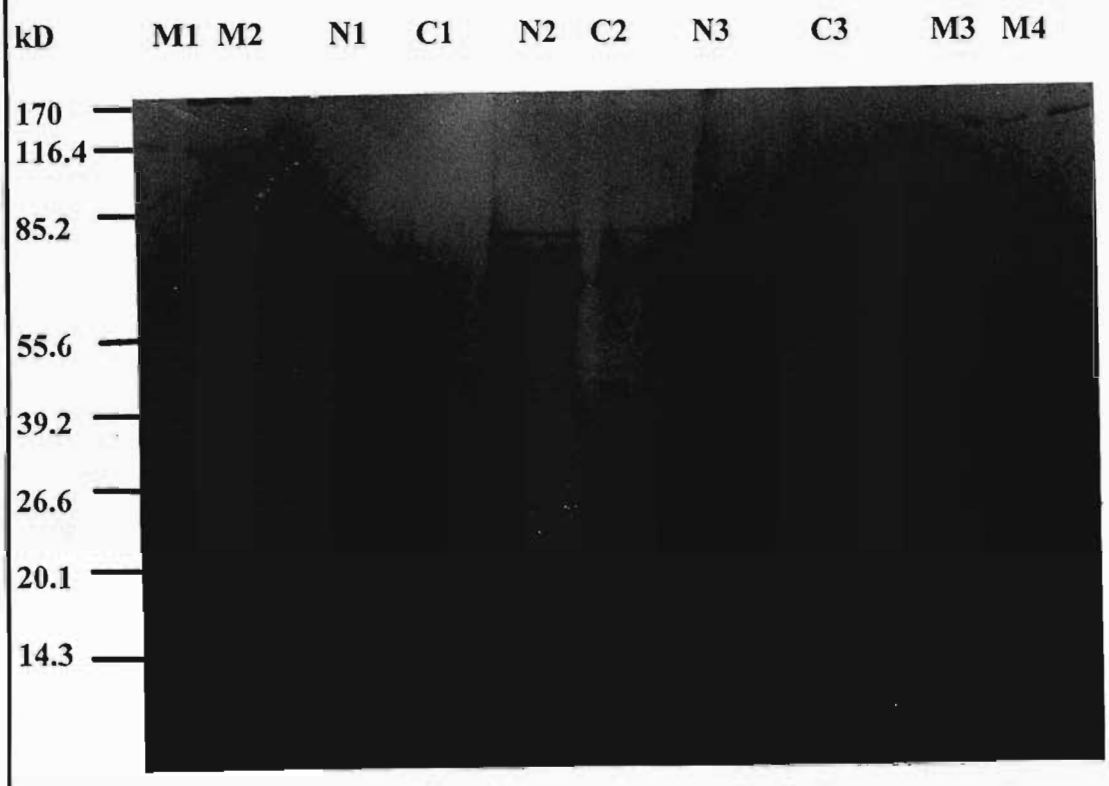


Figure 3.3: 1-D Polyacrylamide gel electrophoresis of soluble proteins of non-cold tolerant (N) and cold-tolerant (C) *Eucalyptus* clones when various amounts of protein were loaded, following method 1 of extraction and precipitation. Amounts loaded were: 100 µg (lanes C1 and N1), 75 µg (lanes C2 and N2) and 50 µg (lanes C3 and N3). Different amounts of molecular markers were also loaded, M1 and M3 (10 µl), M2 (15 µl) and M4 (20 µl). The gel was run for 3 h and stained with 0.125% (w/v) Coomassie Blue, 50% (w/v) methanol and 10% (v/v) acetic acid. Protein sizes are indicated in kD.

ii) Precipitation - quantification of protein

Unlike method 1, method 2 of protein precipitation (section 3.2.4) gave visible pellets. However, with the resuspension buffer used previously in method 1 [0.2 M Tris-HCl (pH 7.5), 1 M sucrose and 0.056 M 2-mercaptoethanol], recovered yield was very low (2.02- 5.08 µg/0.1 ml). This was attributed to inability to resuspend the pellet in the buffer and therefore a number of resuspension strategies were investigated (Table 3.7). Of the treatment that involved a protein pellet being resuspended in 6.31 M DMSO solvent for a while, and then an equal amount of 0.24 M Tris-HCl (pH 7.5), 2.56 M 2-mercaptoethanol and 0.46 M glycerol is added, gave comparatively good results with almost 100 % pellet protein recovery (i.e. resuspension of the pellet) (Table 3.7).

iii) Effect of amount of TCA used for precipitation on % protein recovery

The results shown in Table 3.7 resulted the selection of the DMSO-containing buffer (buffer 5 in Table 3.7) as the better one for pellet resuspension. However, from previous experiments (Fig. 3.2), there was an indication that the amount of TCA used in the precipitation step might affect percentage recovery. Hence, an experiment was designed to investigate this: 40 µg of protein were precipitated with 120, 180 and 250 µl TCA. (Results shown in Table 3.8). Percentage recovery was determined after the first and the second washing of pellet with acetone (Table 3.8). The effect of acetone washing was also investigated (Table 3.8) as wash one and two, respectively. This was done in order to check for any protein loss during the washing step. The

results in Table 3.8 show that after the second wash, the percentage protein recovery went down from 100% to a 80 - 90% regardless of the amount of TCA used. As a result, a gel was run in order to investigate if the protein sample from the first wash was pure enough for use in SDS-PAGE. If the sample was not pure enough, band streaks would have resulted. The results of the gel showed that no band streaking was caused and therefore the second wash was not necessary. These results also indicated that a loading sample of 50 μg of protein to the gel was adequate to produce a clear polypeptide resolution profile (Fig.3.4).

Table 3.6: Effect of type of grinding procedure used to extract leaf proteins. The first grinding procedure involved the use of a Turrax whilst the second procedure changed completely into a use of a mortar and a pestle for grinding in liquid nitrogen. The values are given as ± SE, n= 6 - 12.

Procedure	mg protein/g fmass
Turrax	10.3 ± 14.1
Grinding in liquid nitrogen	1.34 ± 1.4

Table 3.7: The effect of resuspension buffers investigated for pellet resuspension after precipitation using method 2. Buffer compositions are given in the first column , level of resuspension in the second column, and percentage recovery in column 3. +++= good; ++= fair; += poor; and -= none, n= 3-12.

Strategy	Buffer composition	Level of resuspension	% Recovery
1	0.2 M Tris-HCl (pH 7.5) 1 M sucrose 0.056 M 2-mercaptoethanol	+ +	48.8
2	0.12 M Tris-HCl (pH 7.5) 1.28 M 2-mercaptoethanol 0.46 M glycerol	+	4.43
3	0.12 M Tris-HCl (pH 7.5) 1.28 M 2-mercaptoethanol 0.46 M glycerol 0.01 M Triton-X-100 0.01 M SDS	+	4.45
4	0.12 M Tris-HCl (pH 7.5) 1.28 M 2-mercaptoethanol 0.46 M glycerol 0.01 M Triton-X-100	+	5.08
5	0.24 M Tris-HCl (pH 7.5) 2.56 M 2-mercaptoethanol 0.46 M glycerol 6.31 M DMSO	+ + +	96.6
6	As 4. (devoid of glycerol)	+ +	52.3
7	100 mM K_2CO_3	+ +	52.3
8	Resuspend in 1 ml Bradford reagent	-	-

Table 3.8: The effect of amount of TCA on protein recovery after each of the washing steps after precipitation. The composition of the buffer was 0.24 M Tris-HCl (pH 7.5), 2.56 M 2-mercaptoethanol, 0.46 M glycerol and 6.31 M DMSO. n=3.

TCA (µl)	After 1 wash		After 2 washes	
	observed (µg/0.2 ml)	% Recovery	observed (µg/0.2 ml)	% Recovery
120	72.2	100	62.5	87.5
180	72.5	100	63.1	88.3
250	74.4	100	63.6	89.0

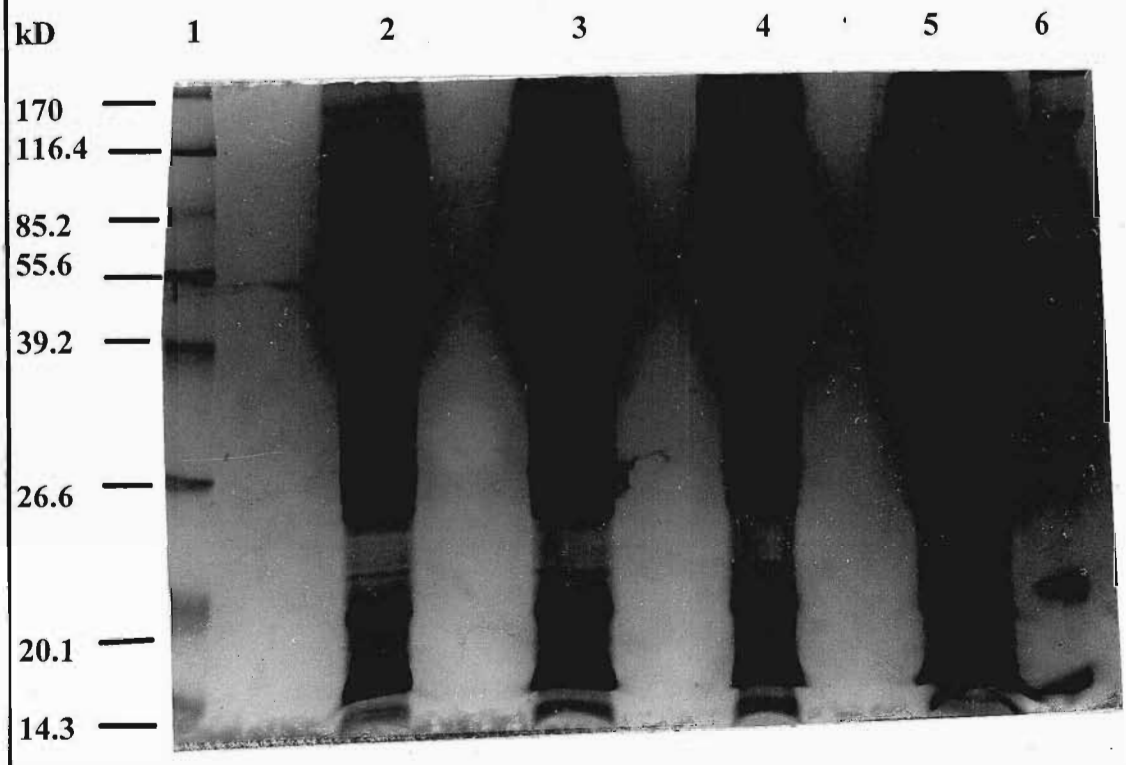


Figure 3.4: 1-D Polyacrylamide gel electrophoresis of soluble proteins of a non-cold tolerant *Eucalyptus* clone. Protein amounts loaded were 40 μg (lane 2), 50 μg (lane 3), 60 μg (lane 4) and 75 μg (lane 5). This gel was run as described in Fig.3.4. The protein sizes are indicated in kD.

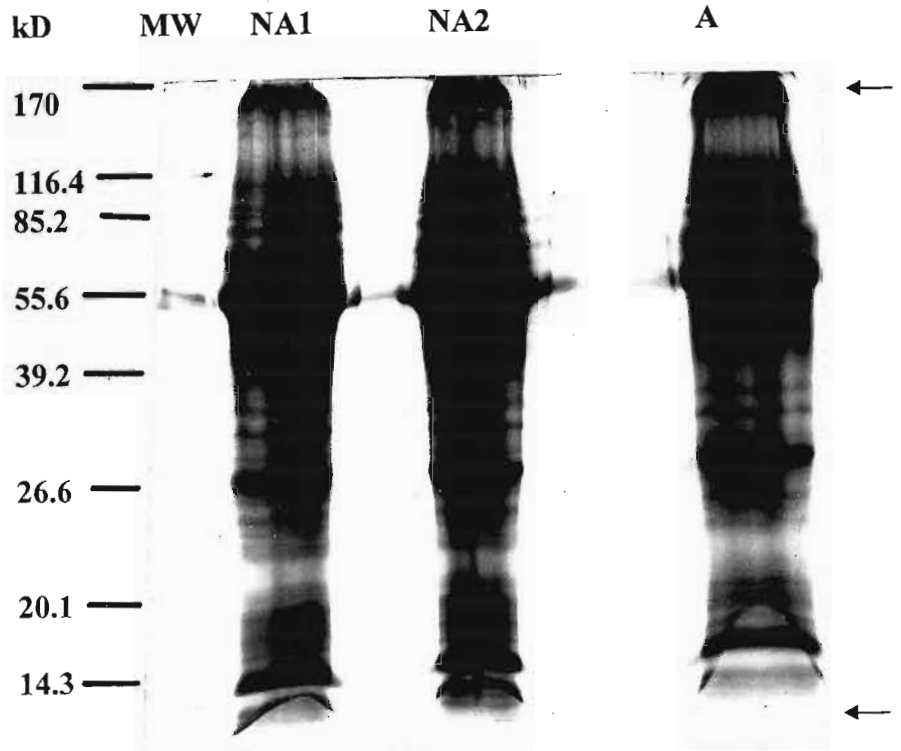
3.3.3 Comparison of polypeptide profiles before and after a cold acclimation

In this study, proteins were extracted from non-acclimated and cold-acclimated (12h at 4 °C) leaf tissue of the non-cold tolerant clone of *E. grandis* (TAG 731) and of the cold tolerant clones *E. saligna* (AS 196, AS 184 and TS 15) and *E. grandis* x *nitens* (GN 1). The extracts were subjected to 1-D gel electrophoresis and the results indicated that discrete changes in protein profiles occurred as a result of the chilling treatment. As a 50 µg protein sample was loaded in each case, variations observed were expected to be due to the qualitative differences in protein bands between the control (non-acclimated) and cold acclimated samples.

In the case of the cold-tolerant clone *E. grandis* x *nitens* (GN 1) (Fig. 3.5), the most apparent changes in polypeptide profiles observed after cold acclimation was the appearance of polypeptides, one in the higher molecular region of 116.4-170 kD and another in a lower molecular weight region of 14.3-20.1 kD. In the non-cold tolerant clone *E. grandis* (TAG 731), no 'new' polypeptides were detected in response to the cold treatment (Fig. 3.6).

When this investigation was repeated using three other cold-tolerant clones *E. saligna* (AS 196, AS 184 and TS 15) the following was observed: The higher molecular weight polypeptide in the region of 116.4-170 kD appeared as a response to the cold treatment in the profiles of *E. saligna* (AS 196 and AS 184) but not in *E. saligna* (TS 15) (Fig. 3.7 and 3.8) respectively. In the case of a low molecular weight polypeptide in the region of 116.4-170 kD, it appeared in all tested

clones (Fig. 3.7; Fig. 3.8 and Fig. 3.9). Unlike the response of plants to heat shock or anaerobiosis where the synthesis of most proteins present, prior to the imposition of the stress, is halted (Guy and Huskell, 1988), virtually all proteins present prior to low temperature exposure continue to be synthesized (Fig. 3.7; Fig. 3.8 and Fig. 3.9).



A

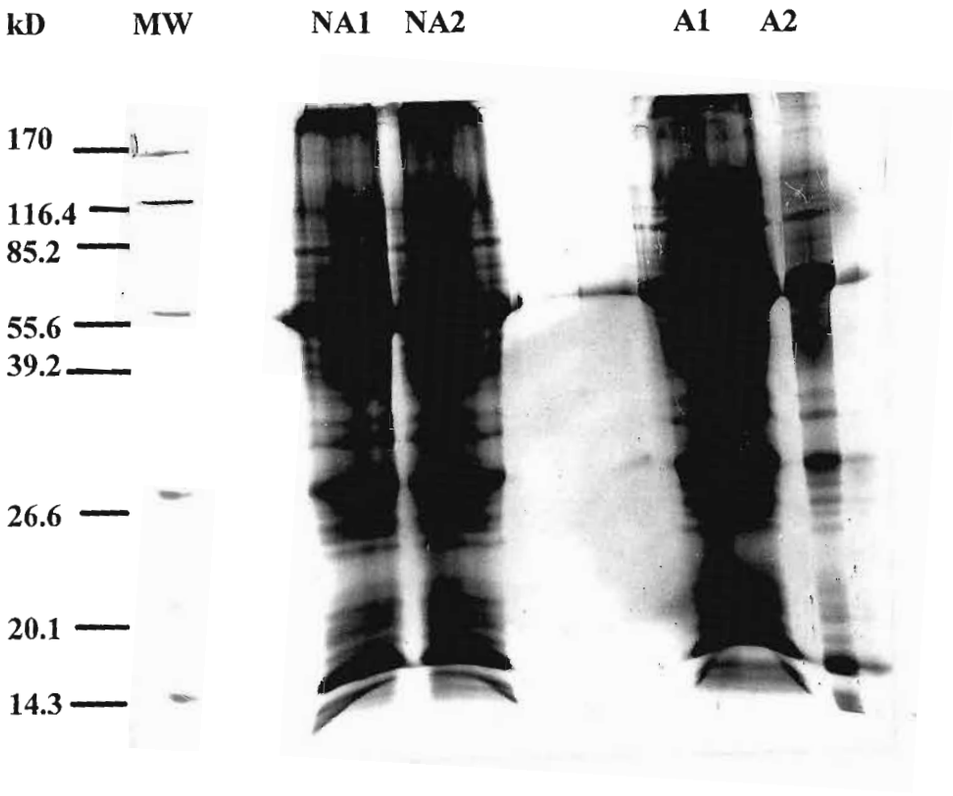
MW (kD) RANGE	NA	A
116.4-170	0	1
85.2-116.4	0	0
55.6-85.2	0	0
39.2-55.6	0	0
26.6-39.2	0	0
20.1-26.6	0	0
14.3-20.1	0	1

B

Figure 3.5: SDS-PAGE profiles of proteins from non-acclimated (NA) and acclimated (A) leaf tissue of cold-tolerant clone *E. saligna* (NG1).

(A) Equal amounts of proteins NA2 and A (50 µg), and NA1 (40 µg) were applied. Molecular weights are indicated in kD. Arrows indicate bands showing proteins that were synthesized in acclimated leaf tissue.

(B) Diagrammatic representation of the number of polypeptides in non-acclimatized and acclimatized sample. Fig 3.5A. Bands were detected with a Densitometric scan.

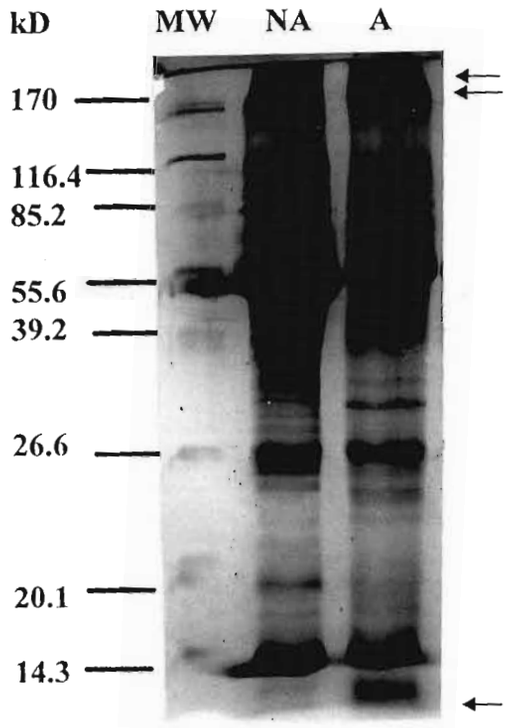


A

MW (kD) RANGE	NA	A
116.4-170	0	0
85.2-116.4	0	0
55.6-85.2	0	0
39.2-55.6	0	0
26.6-39.2	0	0
20.1-26.6	0	0
14.3-20.1	0	0

B

Figure 3.6: SDS-PAGE profiles of proteins from non-acclimated (NA) and acclimated (A) leaf tissue of non-cold tolerant clone of *E. grandis* (TAG 731). (A) Equal amounts of proteins NA1 and A1 (40 µg), NA2 and A2 (40 µg) were applied. Molecular weights are indicated in kD.
(B) Diagrammatic representation of the number of polypeptides in Fig 3.6A. Bands were detected with a Densitometric scan.

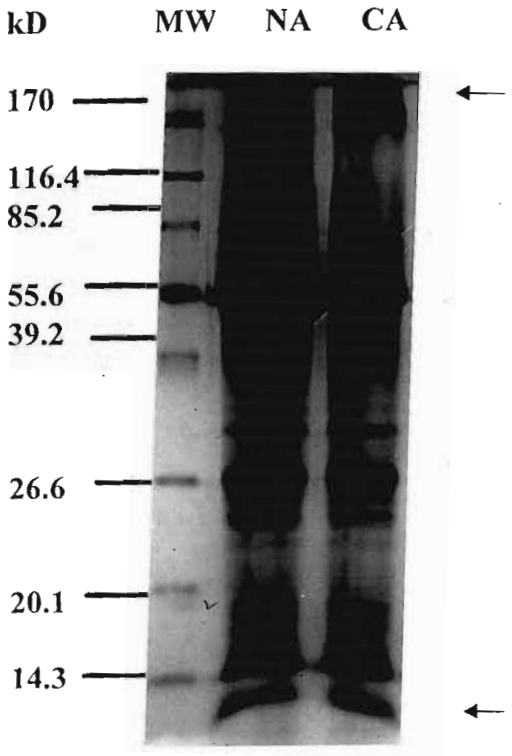


A

MW (kD)	NA	A
116.4-170	0	2
85.2-116.4	0	0
55.6-85.2	0	0
39.2-55.6	0	0
26.6-39.2	0	0
20.1-26.6	0	0
14.3-20.1	0	1

B

Figure 3.7: SDS-PAGE profiles of proteins from non-acclimated (NA) and acclimated (A) leaf tissue of cold-tolerant clone *E. saligna* (AS 196). (A) Equal amounts of proteins NA and A (50 µg) were applied. Molecular weights are indicated in kD. Arrows indicate bands of proteins that were synthesized in acclimated leaf tissue.
(B) Diagrammatic representation of the number of polypeptides in Fig 3.7A. Bands were detected with a Densitometric scan.



A

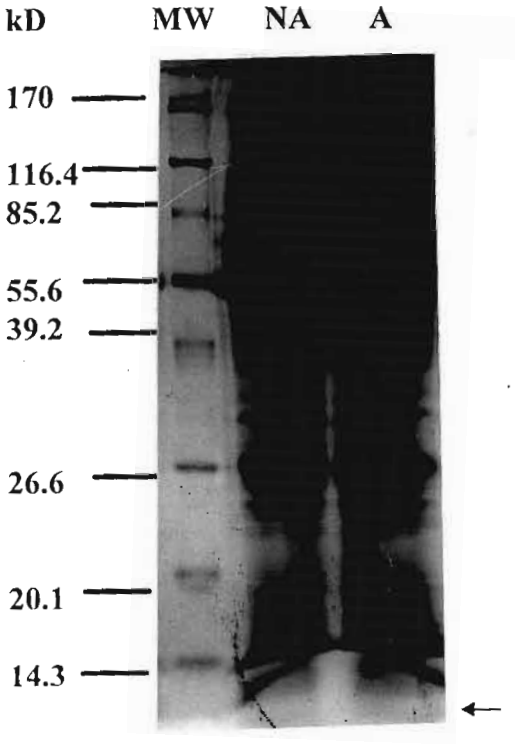
MW (kD)	NA	A
116.4-170	0	2
85.2-116.4	0	0
55.6-85.2	0	0
39.2-55.6	0	0
26.6-39.2	0	0
20.1-26.6	0	0
14.3-20.1	0	1

B

Figure 3.8: SDS-PAGE profiles of proteins from non-acclimated (NA) and acclimated (A) leaf tissue of cold-tolerant clone *E. saligna* (AS 184).

(A) Equal amounts of proteins NA and A (50 µg) were applied. Molecular weights are indicated in kD. Arrows indicate bands of proteins that were synthesized in acclimated leaf tissue.

(B) the number of polypeptides in Fig 3.8A. Bands were detected with Densitometric scan.



A

MW (kD)	NA	A
116.4-170	0	0
85.2-116.4	0	0
55.6-85.2	0	0
39.2-55.6	0	0
26.6-39.2	0	0
20.1-26.6	0	0
14.3-20.1	0	1

B

Figure 3.9: SDS-PAGE profiles of proteins from non-acclimated (NA) and acclimated (A) leaf tissue of cold-tolerant clone *E. saligna* (TS 15).

(A) Equal amounts of proteins NA and A (50 μg) were applied. Molecular weights are indicated in kD. Arrows indicate bands of proteins that were synthesized in acclimated leaf tissue.

(B) Diagrammatic representation of the number of polypeptides in Fig 3.9A. Bands were detected with Densitometric scan.

3.4 DISCUSSION

It has been shown that dormancy development, cold acclimation, and/or N_2 storage in woody plants involve changes in gene expression (Coleman *et al.*, 1991; Arora *et al.*, 1992). This study indicates that cold acclimation in *Eucalyptus* leaves of cold-tolerant clones is associated with the appearence/synthesis of one or two polypeptides. Cold-tolerant *E. saligna* clones (AS 196, AS 184, TS 15) and *E. grandis* x *nitens* (GN 1), chilled in the dark at 4 °C for 12h, exhibited discrete changes in leaf protein synthesis during cold acclimation. Arora *et al.* (1992) have reported that plants subjected to artificial tolerance induction seldom achieve levels of tolerance comparable to those induced under natural conditions. It is therefore acknowledged in the present study, that tolerance levels, as displayed by polypeptide synthesis, might not have been as extreme as those occurring under natural conditions. Since this study evaluated effect cold stress on polypeptides using laboratory temperature regimes, it is suggested that future research should aim at utilising material from field conditions.

Unlike the response of plants to heat shock or anaerobiosis where the synthesis of most proteins present, prior to the imposition of the stress, is halted (Guy and Huskell, 1988), virtually all proteins present prior to low temperature exposure continue to be synthesized (Fig. 3.5; Fig. 3.7; Fig. 3.8 and Fig. 3.9).

Arora and Wisnieski (1995) reported the accumulation of 19 kD and 25 kD polypeptides followed by a subsequent decrease during de-acclimation and the results of the present investigation agree with their findings that a group of low molecular mass polypeptides are apparent in cold-acclimated samples but not in non-acclimated and de-acclimated leaf tissues (Fig.3.5; Fig. 3.6; Fig. 3.7; Fig. 3.8; Fig. 3.9). Again, Guy *et al.* (1988) reported that spinach leaf tissue exposed to 5 °C contained two high molecular mass cold-acclimation polypeptides (CAPs) of 85 kD and 160 kD not found in non-acclimated leaf tissue. The same authors again reported that cold-acclimated citrus leaf tissue also contained a polypeptide of 160 kD not found in non-acclimated citrus leaf tissue. The similarity in molecular weight and isoeletric point between the CAP 160 kD of citrus and spinach suggested that these widely unrelated species may synthesize the same protein in response to exposure to low temperature. The results obtained in the current study revealed the appearance of CAPs in the region of 14.3-20.1 kD and 116.4-170 kD being the most consistent in all experiments. Therefore, it may be assumed that the 160 kD polypeptide found in citrus and spinach (Guy *et al.*, 1988) might be similar to the one found in the current study in *Eucalyptus* (Fig. 3.5; Fig. 3.7; Fig. 3.8) in the region of 116.4-170 kD. Studies conducted by Sarhan and Perras (1987); Perras and Sarhan (1988) associated the accumulation of a high molecular weight polypeptide of 200 kD with cold hardening of etiolated wheat seedlings. Both those authors found that the induction of the 200 kD protein in the early stages of cold hardening process suggested that low temperature induced protein might be

required for the acquisition, the development and/or the maintenance of increased freezing resistance in wheat. The soluble protein patterns also revealed that eight other peptides, ranging from 31 kD to 180 kD, increased in all cultivars upon low temperature exposure. The 180 kD polypeptide may be also similar to the one occurring in the region of 116.4-170 kD in this study.

Although increases in existing proteins and the appearance of new proteins during cold acclimation have been widely documented (as discussed above), metabolic adjustment to low temperature is not well understood (Arora and Wisnieski, 1995). The data presented here indicate that exposure to non-freezing low temperatures caused changes in the pattern of protein synthesis of cold-tolerant *Eucalyptus* leaf tissue. What is not known is whether these polypetides were produced *de novo* (i.e. gene is transcribed and translated) or not (the mRNAs are present and translocation occurs in response to cold).

The limitations of the present study need to be pointed out. Firstly, more clones known to be cold-tolerant and more that are non-cold tolerant have to be tested. This will help provide evidence that polypeptides that are newly synthesised during cold acclimation are causally related to the development of cold resistance. However, such a causal role would appear to be plausible from the parallel increases in the ability to synthesize proteins at low temperature and in the ability to survive freezing temperature. The availability of cold-tolerant-specific clones

could then be used as further probes to elucidate the significance of the biochemical and developmental changes through release of specific polypeptides that a plant undergoes to attain freezing tolerance.

Secondly, this present study does not provide definitive or conclusive evidence for the transcriptional regulation of gene expression during cold acclimation. Hence, results obtained using SDS-PAGE pattern should be confirmed further using other known techniques. There have been a number of studies to identify novel polypeptides associated with the induction of freezing tolerance the synthesis of which is under direct genetic control. The general approach is to label with ^{35}S-methionine, ^{14}C-methionine, or ^{14}C-leucine throughout the hardening period and analyse the results by fluorography of one- or two-dimensional SDS-gels (Cooper and Ort, 1988; Ghosh *et al.*, 1989; Hon *et al.*, 1994). Immunoblotting and *in vitro* translation can also be used to identify novel polypeptides and polypeptides that increase during low-temperature acclimation. Immunoblotting further identifies decreases in polypetides with certain molecular weights (Mohapatra *et al.*, 1987). In future, the discovery of anti-freeze protein intrinsically produced by a cold-tolerant plant may prove important to forestry for two reasons. Firstly, conventional breeding programmes have failed to improve frost resistance in crop because physiological markers specific for frost tolerance are not yet available. Secondly, isolation and characterization of genes for anti-freeze protein may provide the targeting information essential

for the successful transformation of freezing-sensitive eucalypts with genes encoding anti-freeze proteins.

3.5 CONCLUSION AND FUTURE PROSPECTS

In this study, cold tolerance of *Eucalyptus* species, *E. grandis* (TAG 731), *E. grandis* x *nitens* (GN1) and *E. saligna* (AS 184, AS 196 and TS 15) was investigated using one-dimensional SDS- PAGE. Two polypeptides, one in the lower molecular region of 14.3-20.1 kD and another of a higher molecular weight in the region of 116.4-170 kD were observed after cold acclimation. These changes in polypeptide profiles were observed in cold-tolerant *E. grandis* x *nitens* (GN1) and *E. saligna* (AS 184, AS 196 and TS 15) but not in a non-cold tolerant species *E. grandis* (TAG 731).

This study indicates that using the developed protocol, the successful screening of *Eucalyptus* cultivars, both cold-tolerant and non-cold tolerant, could be achieved in relatively short time. This developed diagnostic test could allow *Eucalypus* cold-tolerant clones to be selected at early stages of plant development. Indeed, the main objective of breeders in the forest research today is to reduce the time needed to obtain new clones by efficient screening of a large population. The cold tolerance diagnosis of *Eucalyptus* leaves applied on either on field-grown or greenhouse plants would also allow a rapid selection of the clones as far as resistance to frost is concerned.

This technique, integrated with other proposed future research strategies (discussed in this work) can be a helpful tool to be used in diagnostic tests in breeding programmes aimed at developing *Eucalyptus* cold tolerance.

CONCLUDING REMARKS

The South African population is increasing at alarming rate: so are the demands for forest products. However, land availability is decreasing and it becomes very important to increase efficiency of land utilisation. One strategy is to be able to select efficient in the clones to plant in each area and to produce sufficient quantities of those specifically selected genotypes for those areas. The present work attempted to develop procedures to facilitate such attempts. Several biotechnological techniques, such as tissue culture, have been found to have direct applications in forestry breeding programmes in South Africa (Watt *et al.*, in press).

Cell, tissue and organ culture techniques may be used for the rapid establishment of clonal and breeding seed orchards, particularly of trees which flower early, thus hastening and facilitating breeding programme. The techniques can also be used for the *in vitro* selection of cell lines possessing selected properties. For example, cold-tolerance can be tested by examining protoplast survival under low temperatures (Teulieres *et al.*, 1989). *In vitro* techniques are at present being applied to eucalypts to achieve genetic transformations and these techniques are likely to play a vital role in future tree-improvement programmes. Commercial clonal propagation of cold-tolerant eucalypts in South Africa is a promising technique in the present

economic climate. In order to achieve this, strategies differing from current hedge coppice technique will have to be effected.

In this study, the effects of low temperatures on *Eucalyptus* cultivars have been considered, particularly in relation to resultant changes in protein expression. A wide range of other environmental factors, both biotic and abiotic, affect plant growth and performance. Acclimatory changes resulting in an increase in tolerance to cold stress often occur and it is possible to investigate changes in gene expression associated with this. It is, of course, a lot harder to demonstrate unequivocally that a particular observed change in gene expression contributes to the survival process. Changes in the expression of large numbers of genes occur due to stress, yet it is probable that only some of these genes are directly involved in stress tolerance. Indeed, it is possible that in some cases the synthesis of a protein indicates sensitivity to a stress rather than being part of a tolerance mechanism. One goal of understanding the mechanism of plant acclimation to environmental stress is to be able to manipulate the response of plants so that areas inhospitable to plants, due to natural or man-made causes, can be exploited. However, the response of gene expression to temperature is very complex and the transfer or manipulation of a single gene may not be sufficient to convert a stress-susceptible genotype into one tolerant of the same stress. Nevertheless, an understanding of gene expression in cold acclimation under cold stress has a potential for the exploitation of these gene products in quantitative screening

systems. The developed diagnostic protocol in this study ensures that *Eucalyptus* cultivars, both non-cold tolerant and cold-tolerant are screened at any stage of development provided adequate leaf material is available to carry out protein extraction.

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