IN VITRO PROPAGATION OF *EUCALYPTUS* CLONES USING A TEMPORARY IMMERSION BIOREACTOR SYSTEM (RITA®)

By

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PREFACE

The experimental work described in this thesis was carried out at the Trahar Technology Centre, Mondi Forests, School of Botany and Zoology, University of Natal, Pietermaritzburg, and School of Environmental and Life Sciences, University of Natal, Durban, from February 2001 to December 2002, under the supervision of Dr J. Finnie and Prof. P.Watt.

The studies have not been submitted in any form to another University and, except where the work of others is acknowledged in the text, are the results of my own investigation.

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We certify that the above statement is correct.

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ABSTRACT

Breeding and clonal programs in South African Forestry industry are designed to provide genetically superior trees to supply the forest product industry. Applied biotechnology, and in particular tissue culture, has been used to increase productivity in *Eucalyptus* clones (genetically superior trees) for trials and clonal hedges for commercial production. Improved growth using bioreactors has been increasingly recognized and the traditional semi-solid culture system was evaluated against a temporary immersion bioreactor (RITA®) system. The temporary immersion bioreactor (RITA®) system was tested across different clones for: ease of initiation into the vessels; multiplication numbers required to achieve production targets; and rooting. In addition costs of the RITA® system were evaluated. Contaminant free shoots in the RITA® system were obtained by initiating shoots on a semi-solid medium and thereafter pre-treating with 0.1 $g.l^{-1}$ Rifampicin in liquid MS medium with visual selection of contaminant free plants. Cultures with fungal contamination were discarded as fungicides used as preventives or curatives measures were found to be ineffective against fungal contamination. Bacterial contamination could be reduced or controlled with the use of 0.1 $g.l^{-1}$ Rifampicin. This however sometimes led to a fungal flush or, if Rifampicin was removed, a flush of bacterial contamination then occurred. Factors such as vessel ventilation, times of immersion and rest, size of vessel, ability to have a liquid substrate rather than a semi-solid substrate, and the physical covering of the plants with the nutrients, led to increased multiplication. Number of explants at the start, medium composition and flush and interval times particularly influenced multiplication. Initiating 50 shoots in a vessel with a flush time of 30 seconds and a rest period of 10 minutes gave the highest multiplication (3.8x) after 14 days. Depending on the clone, various media tested had different effects on multiplication. However, MURASHIGE & SKOOG (1962) medium with the following added: 0.1 $g.l^{-1}$ Biotin and Calcium pantothenate; 0.2 $mg.l^{-1}$ BA; 0.01 $mg.l^{-1}$ NAA; and 25 $g.l^{-1}$ sucrose (M1 medium) for both cold-tolerant and sub-tropical clones gave the highest average multiplication after 14 days (5.63x). Maximum shoot multiplication was achieved over 14 to 21 days. After 21 days the nutrients were depleted and the plants began to senesce by day 28. The time period for multiplication in the RITA® system was shorter than for *in vitro* propagation on semi-solid medium, with improved multiplication in half the time using the RITA® system. Nutrients from the media were utilized at different rates in the two systems. Plants from the RITA® system were superior in quality and this had a positive effect on rooting.

The size of the shoot was important for rooting and thus elongation media were tested prior to rooting, with MS and ½ MS giving the best elongation. For rooting in the RITA® system, 1 $mg.l^{-1}$ IBA for two cold-tolerant and one sub-tropical clones gave an average of 66 % normal rooting in the vessels. The type of media used prior to rooting affected rooting and acclimatization percentages. M1 media for 14 days transferring to MS media for 14 days and then placement onto RM media for a further 14 days gave the highest rooting percentage (55 %) after 28 days in the greenhouse. The period of time that the plants were exposed to a particular media played a role in rooting, as did the size of the plants, with bigger shoots (three to seven centimeters) resulting in better rooting. Sub-tropical clones showed no differences in rooting percentages between the semi-solid and the RITA® system rooting environments. However with the cold-tolerant clones rooting was substantially improved with the RITA® system. The plants produced in the RITA® system were of a superior quality and acclimatized more readily than those grown on the semi-solid system. The costs involved in producing plants in the RITA® system were lower, as more plants were produced from the medium in shorter time. Although the initial outlay of vessels for the RITA® system was high, it was offset by reduced labour and media cost, together with significantly higher rooting and survival percentages, thus making the RITA® system a very cost effective option for *in vitro* propagation of *Eucalyptus* clones.

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LIST OF ABBREVIATIONS

μ	- Micro
%	- Percentage
®	- Registered
°C	- Degrees centigrade
$\mu mol.m^{-2}.s^{-1}$	- Micro moles per meter squared per second
µS	- Micro siemen
B	- Boron
BA	- 6-Benzylaminopurine
Ca	- Calcium
cm	- Centimeter
Cu	- Copper
EC	- Electrical conductivity
Fe	- Iron
$g.l^{-1}$	- Grams per litre
GN	- *Eucalyptus grandis* x *Eucalyptus nitens*
GU	- *Eucalyptus grandis* x *Eucalyptus urophylla*
IAA	- Indole-3-acetic acid
IBA	- Indole-3-butyric acid
K	- Potassium
Kpa	- Kilopascal
M	- Molar
Mg	- Magnesium
min	- Minutes
ml	- Millilitres
Mn	- Manganese
MS	- Murashige & Skoog (1962) medium
N	- Nitrogen
NAA	- α-Napthaleneacetic acid.
NH	- *Eucalyptus nitens* natural hybrid
P	- Phosphorous
pH	- Hydrogen ion concentration
rpm	- Revolutions per minute
s	- Seconds
s.d.	- Standard deviation
TAG	- Transvaal first generation *Eucalyptus grandis*
Tween 20	- Polyoxyethylene sorbitan monolaurate
vs.	- Versus
x	- Times

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INTRODUCTION

Eucalyptus species and hybrids are important plantation trees throughout the world, including South Africa. They are used for a wide variety of purposes and a range of species such as *Eucalyptus diversicolor, E. dunnii, E. fastigata, E. grandis, E. macarthurii, E. nitens, E. paniculata* as well as many hybrid species are planted (DENISON & KIETZKA, 1993; SMITH, 1996). Different species are used for production of: charcoal; domestic and industrial energy; essential oils; honey; sawn timber and tannin (HILLS & BROWN, 1978; McCOMB & BENNETT, 1986; TURNBULL, 1991). In South Africa companies (mainly Mondi Forests and Sappi) grow *Eucalyptus* primarily for the pulp, paper and packaging industry. However *Eucalyptus* is also grown for woodchip export and mining timber (SMITH, 1996). The South African forestry industry plays an exceedingly important role in the national economy and for employment (Table 1).

Table 1. Afforested areas, output, earnings and the importance of South African forestry (OWEN & VAN DER ZEL, 2000)

Indicator	**South Africa**
Afforested area (plantations only)	1.5 million hectares
Annual harvest	16 million cubic meters
Output (forestry and products)	R 13 billion
Export earnings	R 6.6 billion
Employment (forestry and processing)	135 000 people
Contributions to the gross domestic product	2%

Due to diverse climatic conditions in South Africa, a variety of *Eucalyptus* species and clones are needed in order to produce appropriately site-matched planting stock in as short a time as possible. Consequently, clonal and breeding programs in forestry companies have been developed and advanced to produce genetically superior trees for the future demand of forest products in this country. In Mondi Forests' *Eucalyptus* clonal program there is increasing focus on selected hybrids (*viz. E. grandis* x *E. urophylla* and *E. grandis* x *E. nitens* hybrids), which are disease resistant, have more homogeneous wood density and withstand stress and climatic conditions (DENISON & KIETZKA, 1993). Due to the varied soils, rainfalls, altitude and climatic conditions (Figure 1) different clones have been developed. The most common hybrid combinations for the sub-tropical areas are *Eucalyptus grandis* crossed with *E. camaldulensis*

(GC), *E. urophylla* (GU) or *E. tereticornis* (GT). For the temperate areas the hybrids produced are *E. grandis* x *E. nitens* (GN), and *E. nitens* hybrids (natural hybrids, NH) (DENISON & KIETZKA, 1993). These hybrids out-perform the pure species on marginal sites, as they are more resistant to diseases, pests, cold, heat and drought (DENISON & KIETZKA, 1993).

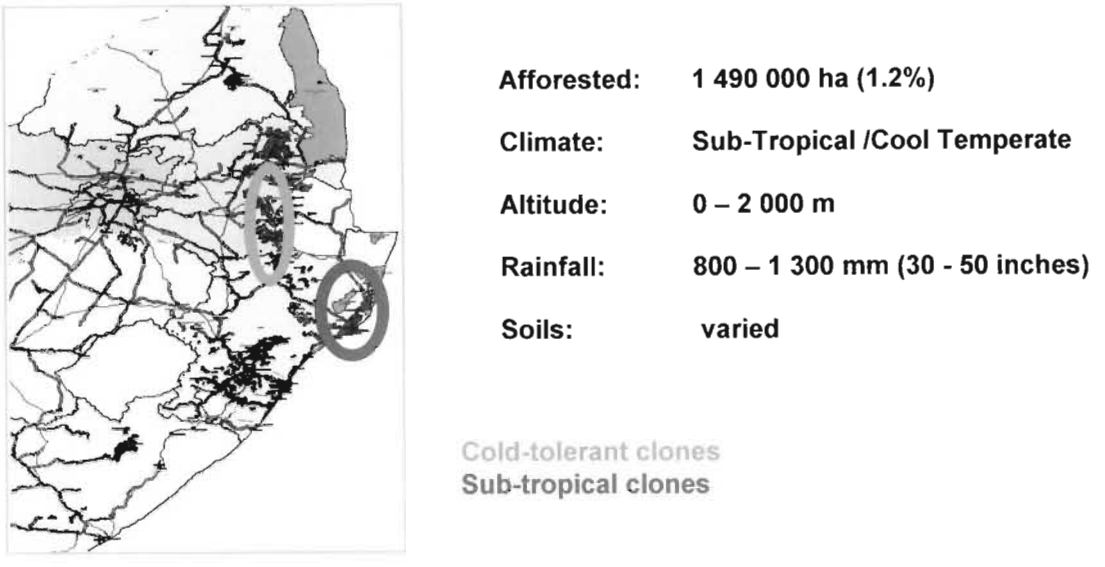


Figure 1. Different clones developed for the different climates, altitudes, soils and rainfall areas in South Africa (Black dotted areas are the afforested areas)

Cold-tolerant clones have superior pulp properties and yields and are thus extremely important (DENISON, 1999). Many of the cold-tolerant clones are however very difficult to root and research is ongoing to improve their rootability. The increasing focus on these selected hybrid combinations of *Eucalyptus* has resulted in increased production targets for breeding, clonal and commercially approved material. Thus the need to produce high yields of better quality plants at a faster rate and lower cost is of vital importance.

Micropropagation has been used commercially for a large number of plant species, including trees, as multiplication of shoots is more rapid than other vegetative methods of multiplication (GEORGE, 1993). Micropropagation together with macro-cuttings have been used to vegetatively propagate large numbers of clones of numerous *Eucalyptus* species and hybrids for clonal hedges. To date, the most common method of micropropagation of *Eucalyptus* involved the proliferation of shoots via a semi-solid system (LE ROUX & VAN STADEN, 1991). While such semi-solid systems have been moderately successful in terms of multiplication yields, it has

become increasingly important to improve productivity and reduce time taken to multiply commercially important material.

At Mondi Forests, *Eucalyptus* plants produced *in vitro* are used to renew or replace macro-hedges and hydroponic mini-hedges (Figure 2).

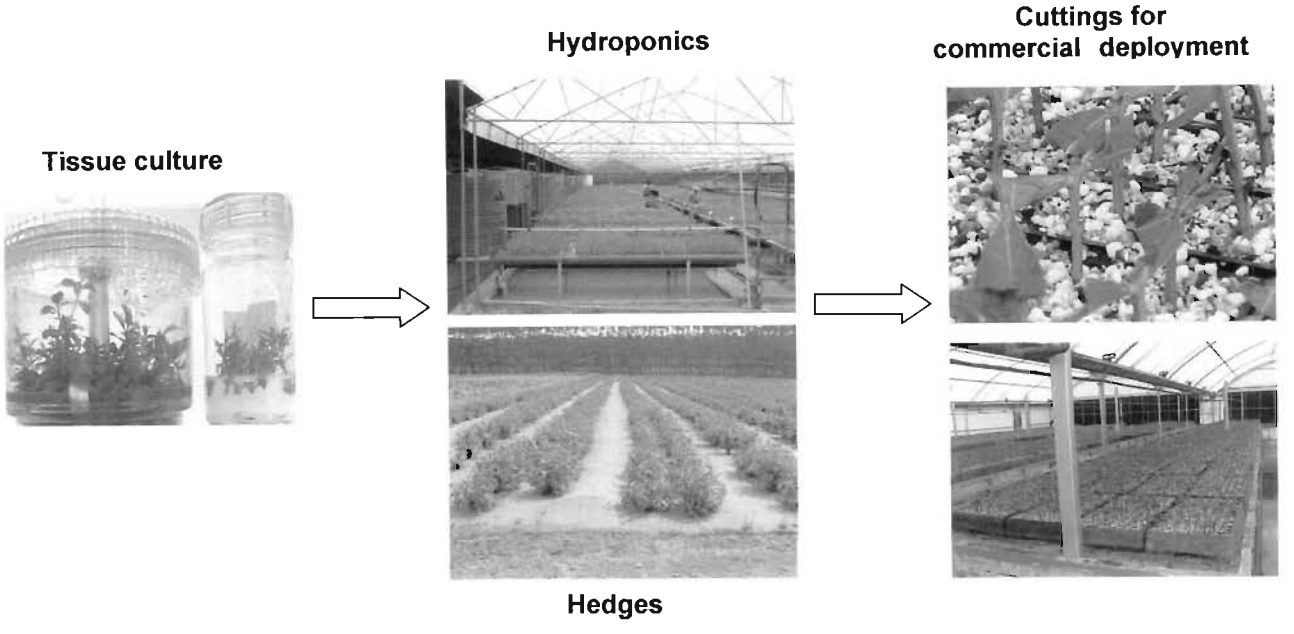


Figure 2. *In vitro* produced plants supply hydroponic hedges and macro-hedges for cuttings to be taken for commercial deployment

Plants are produced *in vitro* using a conventional semi-solid method of axillary shoot production. As reports became available on liquid bioreactor systems that showed improved multiplication and decreased labour costs, it appeared that this system might be suitable for the *in vitro* culture of *Eucalyptus*. A pilot study was built using locally available components to verify if it was possible to enhance multiplication and reduce costs of *Eucalyptus* production utilizing a liquid system (Appendix 1). It was found that, although there was an increase in multiplication and a shorter handling time, the plants were hyperhydric and deformed due to complete submersion. A more suitable method of liquid culture was required to obtain the desired results.

In the last few years, reports in the literature have shown that temporary immersion bioreactor systems, such as RITA®, have numerous advantages over the semi-solid methods. Temporary immersion systems combine the advantages of solid and liquid media, in particular having intermittent total availability of nutrients, but still allowing the plants to grow in an air space.

Using the RITA® system, ESCALONA, LORENZO, GONZÁLES, DAQUINTA, GONZÁLES DESJARDINS & BORROTO (1999) found that temporary immersion increased multiplication rates for *in vitro* shoots of pineapple. AKULA, BECKER & BATESON (2000) reported that the immersion frequency and immersion time impacted on multiplication rates of tea. ETIENNE, LARTAUD, MICHAUX-FERRIÈRE, CARRON, BERTHOULY & TEISSON, (1997) found that using the RITA® system increased the root development of *Hevea brasiliensis.* Examples of other advantages listed by various authors include improved micropropagule quality, reduced consumable costs, reduced labour costs (ETIENNE *et al.* 1997; BORROTO, 1997), better leaf development, reduced hyperhydricity and minimized asphyxiation of tissue (AITKEN-CHRISTIE, KOZAI & TAKAYAMA, 1995). Further, plants from the temporary immersion system have been found to be more suitable for acclimatization and development towards photoautotrophy (AITKEN-CHRISTIE *et al.* 1995).

The demand by the forestry industry for improved productivity (of superior trees) through biotechnology is ever increasing. Therefore it is of great importance to optimise production of *Eucalyptus* at the lowest possible cost. This study was undertaken to investigate if the temporary immersion liquid system (RITA®) could facilitate faster production of better quality *Eucalyptus* clones in a more cost-effective manner. In this context, the aims of this study were to:

- obtain contaminant free plants in the RITA® vessels
- determine if increased multiplication could be achieved by using the temporary immersion system (RITA®)
- determine if increased rooting on sub-tropical and especially cold-tolerant clones could be obtained using the RITA® system
- obtain better quality plants using the temporary immersion system
- compare the value of the temporary immersion system with the conventional semi-solid system in terms of yields, costs and application to the *Eucalyptus* plantation industry.

Chapter 1. Literature Review

1.1. Introduction

The practice of vegetative propagation of plants is as old as the cultivation of plants itself. With the development of techniques of cell and tissue culture a new dimension was added to the age-old conventional practices of propagation (MINOCHA, 1980). Plant tissue culture may be defined as the culture of all types of plant cells, tissues and organs under aseptic conditions (SMITH & DREW, 1990). It is a proven technology for the *in vitro* production of large numbers of genetically identical pathogen-free plants (DEBERGH & READ, 1991; AITKEN-CHRISTIE *et al.*1995; KURATA, 1995).

Tissue culture is being used in many fields of plant biotechnology such as: breeding/selection; vegetative propagation; maintenance of pathogen-free (indexed) germplasm; secondary metabolite production; cryopreservation; and clarification of physiological, biochemical and genetic processes with respect to embryogenesis, organogenesis or growth and development of cultures or plant material *in vitro* (SMITH & DREW, 1990; KOZAI & SMITH, 1995; ZIMMERMANN, 1997). Different techniques of *in vitro* culture are utilized to obtain plants, dependent on the requirement (SMITH & DREW, 1990). Shoots produced *in vitro* from axillary or adventitious origin are one of the most common types of tissue produced by the widest range of species. There is a huge variety in plant shape, leaf shape and texture, growth habit, growth rate and method of multiplication between species but all are capable of growth and multiplication and many are now commercially produced by different tissue culture methods (AITKEN-CHRISTIE *et al.* 1995).

There are two main plant crop products that have benefited from tissue culture technology. Firstly, crops such as timber, cereals, vegetables and fruits are generally products that have been cultivated for a long time, meet well-defined industry parameters and are not usually subject to consumer whims and, secondly, the specimen plant which is generally sold in nurseries to gardeners, has been a short time on the market and is affected by consumer trends (WILSON, 1995). In forestry, elite material will either be a selected phenotype or the plant will be a bearer of elite seed, which will be used as starting material (DEBERGH & READ, 1991).

1.2. *In vitro* culture of *Eucalyptus*

The genus *Eucalyptus* L'Her (Myrtaceae) consists of fast-growing trees which originate from Australia. *Eucalyptus* is represented by about 8000 species and hybrids, some of which are commercially important for timber, essential oil and pulp (GUPTA & MASCARENHAS, 1983). *Eucalyptus* species and hybrids are important plantation trees throughout the world, including South Africa, as they are used for a wide variety of products (DENISON & KIETZKA, 1993). *Eucalyptus* is naturally regenerated by seed, which usually results in highly variable progeny. To cope with the ever-increasing demand for wood (for fuel and industrial purposes) intensive forestry practices are necessary to develop superior clones for mass propagation. As seedlings have a large variation in pulping yields clonal propagation has been undertaken (LAKSHMI SITA, 1993). Genetic fidelity of clonal material is important in clonal forestry programs (AHUJA, 1993). Variations in morphology between provenances are evident but variations within clonal lines are small. This indicates that micropropagation processes can produce very uniform trees when compared with seedlings. Uniformity of trees is an important feature for commercial production of *Eucalyptus* trees. Pulp, paper and saw mill operators prefer material of similar measurements and quality for economic processing (BELL, VAN DER MOEZEL, BENNETT, McCOMB, WILKINS, MARSHALL & MORGAN, 1993). A variety of *Eucalyptus* species and clones are needed in South Africa, as there are diverse soil types, altitudes and climatic conditions. Consequently, various clones are produced in as short a time as possible for appropriately site-matched planting stock. In forestry companies there is an increasing focus on selected hybrids (*viz. E. grandis* x *E. urophylla* and *E. grandis* x *E. nitens*), which are disease resistant, have more homogeneous wood and fibre properties and withstand stress and harsh climatic conditions (DENISON & KIETZKA, 1993).

Since the 1930s tissues from woody plants have been cultured (AHUJA, 1993). Juvenile explants, such as embryos, cotyledons, hypocotyls or bud explants from seedlings are considered more responsive to *in vitro* regeneration than are tissues from mature trees. Thus juvenile explants have been extensively employed for the clonal propagation of woody plants (AHUJA, 1993). Micropropagation of juvenile material has a disadvantage in that it is difficult to determine how the seedling will perform on reaching maturity, and thus cloning of mature trees is generally preferred (AHUJA, 1993; LAKSHMI SITA, 1993). Procedures to obtain juvenile

material from mature plants are of considerable importance (HACKETT & MURRAY, 1993). The juvenile tissues of mature trees (stump sprouts, sprouts from pruned trees) are the best explants if regeneration of plants is to be achieved. Selection of the appropriate explant is critical if the optimum number of rootable shoots is to be obtained (THORPE & KUMAR, 1993). The induction of adventive embryos, and adventitious shoots and roots is greatly influenced by the maturation state of the tissue used for the primary explant (HACKETT & MURRAY, 1993). Obtaining juvenile material from mature plants can be approached in two ways: using juvenile parts of the mature plant or by rejuvenation of mature parts of the plant. The term rejuvenation implies a reversal of the maturation process (HACKETT & MURRAY 1993).

Explants from tree species are generally difficult to grow and differentiate *in vitro*. Nevertheless, callus and organ culture have been employed with varying degrees of success for the micropropagation of a number of woody plants (AHUJA, 1993). Long-term callus cultures invariably exhibit genetic variability. Organ cultures and meristem cultures are more stable and involve minimum genetic risk in clonal propagation. Organogenesis involves differentiation of micro-shoots and roots at different time periods during plantlet development. Usually micro-shoots are induced on tissues using cytokinin-enriched medium, and subsequently the micro-shoots are rooted in an auxin-enriched medium to give rise to plantlets. Organogenesis is greatly influenced by the genotype, physiological state of the explants, age of the explants and the *in vitro* environment (AHUJA, 1993). AZIM, NOIN, LANDRÉ, PROUTEAU, BOUDET & CHRIQUI (1997) observed that there were differences in the ability of *Eucalyptus globulus* seeds and clones to regenerate buds. They determined that different hypocotyls produce different numbers of buds but these buds then remain true to type through the tissue culture process. Micropropagation via somatic embryogenesis involves the development of embryos from embryogenically competent somatic cells *in vitro* (AHUJA, 1993). Somatic embryogenesis has been undertaken for some *Eucalyptus* species with a relatively low level of success (WATT, BLAKEWAY, CRESSWELL & HERMAN, 1991; TERMINGONI, WANG & HU, 1996). Shoot multiplication by enhanced axillary and terminal meristem culture, from seedling as well as mature material is the most commonly exploited and successful technique in *Eucalyptus* species (LAKSHMI SITA, 1993). Each leaf has an axillary bud in its axil, which has the potential to develop into a shoot. In nature, these buds remain dormant for various periods,

depending on the growth pattern and environmental conditions. However, by culturing nodal segments on media containing appropriate concentrations of cytokinins, it is possible to break this dormancy with the subsequent development of multiple shoots from nodal segments of aseptic plantlets (LAKSHMI SITA, 1993).

Shoot cultures are a means of long-term plant cultivation in an organized condition. In contrast to callus cultures, they retain their regenerative capacity over long periods. Hence they are particularly appropriate as stocks for the clonal propagation of crops, ornamentals and trees with a high multiplication rate and sufficient genetic stability. A further negative aspect of callus culture is that somaclonal variation may occur in long-term cultures (AHUJA, 1993; SKIRVIN, ABU-QAOUD, SRISKANDARAJAH, & HARRY, 1993). Somaclonal variation is defined as genetic variation observed among progeny of plants regenerated from somatic cells cultured *in vitro*. Although somaclonal variation is not wanted in most cultures it can be utilized for breeding by: selection for cold resistance; disease resistance; herbicide resistance; etc. (SKIRVIN *et al.* 1993).

LAKSHMI SITA (1993) and AZIM *et al.* (1997) reported on different *Eucalyptus* species (*E. grandis, E. citriodora, E. camaldulensis, E. globulus, E. Torreliana, E. territicornis*) being produced by various workers using different methods of propagation. In the review undertaken by LE ROUX & VAN STADEN (1991) it is apparent that the most common method of micropropagation of *Eucalyptus* is by the proliferation of shoots via a semi-solid system. This review gives an extensive overview of the different techniques and the *Eucalyptus* species being produced by *in vitro* methods. WATT, BLAKEWAY, MOKOTEDI & JAIN (2002) have discussed at length the historic perspective of the different processes, species and clones of *Eucalyptus* in production *in vitro* up to 2001. Their review showed that the most commonly used explants in developing micropropagation protocols were seedlings and then axillary buds from field grown plants. However, the propagation yields from seedlings have been reported to be higher than those from axillary buds. WATT *et al.* (2002) also described the current applications of mass production of selected genotypes. They stated that in 2000 Shell Forestry Technology Unit patented a bulk-up liquid system, which was tested across 150 *Eucalyptus* genotypes, and this system offered faster deployment of elite germplasm together with rapid access to and multiplication of germplasm. In a report by BAYLEY & BLAKEWAY (2002) the advances and

deployment obtained by genetic gain (using genetically improved material) are described using advanced multiplication techniques and in the quality of the material and its effect on the establishment of *Eucalyptus* crops. The multiplication (macro- and micropropagation) techniques are a means of amplifying improved material being produced by extensive breeding programs. The use of tissue culture technology for the production of trees for forestry has received considerable attention over the last few decades.

With the importance of forestry (in South Africa and other countries) many articles have been written on the applications of *in vitro* culture of *Eucalyptus* (BONGA, 1977; SOMMER, 1981; MASCARENHAS & MURALIDHARAN, 1989; THORPE, HARRY & KUMAR, 1991; HAMMATT, 1992; DENISON & KIETZKA, 1993; LAKSHMI SITA, 1993; WATT, DUNCAN, ING, BLAKEWAY & HERMAN, 1995; WATT, BLAKEWAY, HERMAN & DENISON, 1997a, WATT, BLAKEWAY, HERMAN & DENISON, 1997b; WATT, MYCOCK, BLAKEWAY & BERJAK, 2000; BAYLEY & BLAKEWAY, 2002; WATT *et al.* 2002). As discussed by all those authors, *in vitro* propagation is an extremely valuable tool with which to produce superior material more rapidly as there is the ability to control the environmental factors, which influence growth and development.

1.3. Control of cultural factors

The control of cultural factors has the advantage of allowing manipulation of plants compared with other methods of plant production. Cultural factors in plant production which require attention through the manipulation of the physical and chemical environments are as follows:

- loss of plants due to contamination
- growth rate of cultures at each stage of culture; variation in size, shape and quality of plants
- loss of plants due to environmental stress during acclimatization
- significant costs in production of plants
- costs related to space required for the different stages (KOZAI & SMITH, 1995).

1.3.1. Contamination

In plant tissue culture, the elimination of microbial contamination from cultures and culture medium at the initiation of culture and the maintenance of an aseptic environment during the culture are of primary importance (KOZAI & SMITH, 1995). Microbial contamination (caused by bacteria, fungi, yeast and insects) in any form is a serious problem in plant tissue culture because of the loss of culture material and subsequent cost implications. The costs due to the loss of plants with microbial contamination in a production tissue culture laboratory can be very high, especially if contamination rates are high in the early stages of culture and these go undetected (LEIFERT, WAITES & NICHOLAS, 1989; PAEK, HWANG & HAN, 1993; HOLDGATE & ZANDVOORT, 1997; LEIFERT & WOODWARD, 1998). Many scientific and commercial laboratories fail to record contamination losses. A level of contamination losses below 2 % per subculture is the minimum required to guarantee successful production (LEIFERT & WOODWARD, 1998).

The objectives behind the elimination of microbial contamination and the maintenance of an aseptic environment are to obtain pathogen free plants and to eliminate or minimize the death or degradation of plants due to microbial contamination during *in vitro* culture (KOZAI & SMITH, 1995). Unacceptable contamination can be discarded at each subculture stage but attempts at elimination of the micro-organisms may be made by surface sterilization of the cultures employing fungicides or antibiotics (HOLDGATE & ZANDVOORT, 1997). Microbial contamination affects the net multiplication rate but may also be severely manifested at the rooting stage by failure to root *in vitro* or *in vivo* and lowering the plant survival rate during acclimatization (COOKE, WAITES & LEIFERT, 1992; HOLDGATE & ZANDVOORT, 1997; LEIFERT & WOODWARD, 1998). Hence it is very important to obtain and maintain micro-organism free cultures. Most problems of contamination arise from inefficient methods for: sterilizing the explants taken from *in vivo* plants; handling aseptic plant material and sterilization of culture vessels, instruments and media (LEIFERT *et al.* 1989; FALKINER, 1997).

Prior to introduction to *in vitro* culture all parent plant material should be inspected for symptoms and treatment should be undertaken (CASSELLS, 1997). In most cases the micro-organisms, which cause microbial contamination and the death of plants *in vitro*, are not

pathogens which may cause diseases of plants in the field or in the greenhouse (KOZAI & SMITH, 1995). It is important to develop a good pre-culture cultivation program and preferably in an insect free, high quality growing environment to reduce the chance of pathogenic infections (DE FOSSARD & DE FOSSARD, 1988; HOLDGATE & ZANDVOORT, 1997). Parent plant material may be pre-treated under running water and soaked in fungicides and/or bactericides prior to surface sterilization to facilitate the elimination of microbes.

Initiation of cultures requires the killing of microbes on the tissue explants without causing phytotoxicity (DE FOSSARD & DE FOSSARD, 1988). The surface disinfection of the explants prior to placement into a tissue culture system is of vital importance. Problems occurring at surface sterilization are due to the disinfectant being inactive or the micro-organisms being protected within the plant tissue which is used as the explants (LEIFERT & WOODWARD, 1998). With poor sterilization methods the greatest loss of tissue may occur at introduction. Surface sterilization of plant material may be accomplished using solutions of sodium hypochlorite, calcium hypochlorite or mercuric chloride together with fungicides or quaternary ammonium compounds (DODDS & ROBERTS, 1985; DE FOSSARD & DE FOSSARD, 1988).

Contamination is not always seen at the culture establishment stage, but may become evident at later subcultures and is then difficult to eliminate (REED, MENTZER, TANPRASERT & YU, 1997). Most micro-organisms are favoured by a culture medium containing sugar and other nutrients. Micro-organisms can quickly increase in the medium during culture, consuming the sugar and nutrients and competing with cultured plants; they may then produce toxic substances resulting in death or degradation of plants *in vitro* (KOZAI & SMITH, 1995). If the *in vitro* plants become more photoautotrophic the growth of micro-organisms may be considerably restricted by the elimination of sugar in the culture medium. The presence of micro-organisms should not cause the death or degradation of plants *in vitro* if the micro-organisms are not pathogens. In the photoautotrophic environment plants and non-pathogenic microbes may be able to co-exist without loss of plant growth rate and quality (KOZAI & SMITH, 1995).

In plant tissue culture bacterial contamination is the most serious form of contamination (KUNNEMAN & FAAIJ-GROENEN, 1988; WATT, GAUNTLETT & BLAKEWAY, 1996). It can be caused by surface and/or endogenous bacteria populations and, in this regard, CORNU &

MICHEL (1987) found that after many months of culture, bacterial contamination occurred on what were apparently healthy cultures. Early detection and prevention of bacterial contamination may be controlled with the use of antibiotics (KUNNEMAN & FAAIJ-GROENEN, 1988; REED *et al.*1997). The use of antibiotics in plant tissue culture can effectively control bacterial contamination without becoming phytotoxic or affecting the growth of the explants (KATZNELSON & SUTTON, 1951). A wide range of antibiotics such as Rifampicin, Streptomycin, Tetracycline, Penicillin and Claforan have been used in controlling such contaminants (Table 1.1). Antibiotics may be incorporated into the medium (prophylaxis) but for this to be effective the pathogen must remain sensitive to the antibiotic. Prophylaxis should be of a short duration and specific (FALKINER, 1997).

With the use of antibiotics, there is a risk that prolonged exposure may increase resistance and phytotoxicity. HUSSAIN, LANE, & PRICE (1994) undertook studies in which they screened 21 micro-organisms for anti-microbial activity against contaminants as alternatives to antibiotics to overcome the problems of antibiotic use. LEIFERT (2000) describes a method for screening for bacterial contamination using hazard analysis critical control points (HACCP), and prevention of contamination at the sources. The HACCP principles and application to tissue culture have been reviewed extensively (LEIFERT & WAITES 1994; LEIFERT & WOODWARD 1998). This method could prove to be a more efficient approach in elimination or control of contamination *in vitro* than the use of anti-microbial substances.

Table 1.1. Different antibiotics used to control bacterial contamination *in vitro*

Antibiotic used	**Effect on bacteria and plant**	**Reference**
Aspergillic acid Aureomycin Clavacin Gliotoxin Penicillin Streptothricin Terramycin Tyrothricin	Aureomycin inhibited growth of bacteria and Terramycin was most effective against *Pseudomonas* sp followed by Streptomycin. The remaining antibiotics had little or no effect against the bacterial contamination	KATZNELSON & SUTTON, 1951
Chloramphenicol Nalidixic acid Phosphomycin Rifampicin Streptomycin	Rifampicin was an effective antibiotic with no phytotoxic effects to plants (plants grew well). The others were not as effective and some had phytotoxic effects to the plants	PHILLIPS, ARNOTT & KAPLAN, 1981
Aminoglycoside	Active against bacteria but chlorosis of the plants occurred.	CORNU & MICHEL, 1987
Gentamicin	Only killed one strain of bacteria tested.	
Rifampicin	Reduction of bacterial contamination occurred. Good for use as a broad-spectrum antibiotic for a woody species (*Prunus avium* L.). Not toxic to plants	
Penicillin	Not as active as Rifampicin or Aminoglycoside	
Tetracyclines	No loss of material but chlorosis occurred, necrocis and death occurred at higher concentrations	
Alcide	40 % survival of the shoots and no contamination at a 10 % solution	DEBERGH & VANDERSCHAEGHE, 1988
Antibiotics against gram positive and gram negative bacteria	Bacteria were isolated from plants and 28 antibiotics and seven mixes of antibiotics were tested. Imipenem/Ampicillin and Imipenem/Penicillin at 5 $mg.l^{-1}$ inhibited bacteria with no toxic effects to the plants	KNEIFEL & LEONHARDT, 1992
Penicillin (1 $g.l^{-1}$) and Streptomycin (1 $g.l^{-1}$)	If these were used for a period of 40 minutes to three hours after disinfection with sodium hypochlorite a 40 % reduction in contamination was achieved but it delayed callus induction and inhibited somatic embryogenesis	TENG & NICHOLSON, 1997
Broad spectrum antibiotics	Reduced bacterial contamination but once removed from the media bacterial infestation recurred	FALKINER, 2000
Antibiotics against gram negative	Unsuccessful, bacteria persisted	LEIFERT, 2000

Bacterial contamination is not the only cause of losses in plant tissue culture. Fungal contamination can cause high losses. One of the main hindrances in the micropropagation of *Eucalyptus spp.* is the difficulty of obtaining aseptic plant tissue from mature field-grown material as the trees have a long life cycle. Fungal contamination in *Eucalyptus spp.* is the single greatest cause of loss during micropropagation (WATT *et al.* 1996). Various fungicides have been used effectively as preventative or as curative measures of controlling fungal contamination in cultures. Benlate® (benomyl) has been found to reduce fungal contamination but it inhibits shoot growth of *Eucalyptus grandis*. Bravo® (chlorothanlonil) at lower concentrations has no effect on *Eucalyptus grandis* plants and is effective against fungal contamination, and Previcure N® (propamocarb hydrochloride) reduces fungal contamination but inhibits shoot growth. Amphotericin B, an anti fungal antibiotic, reduces fungal contamination (WATT *et al.* 1996).

Contamination becomes even more critical in scaled-up automated systems such as bioreactors and robotics because larger volumes of plant tissue are at risk at any one time. The chances of introducing contaminants during the setting up of a bioreactor may be high. Close observation of the status of the tissue or prescreening is necessary as well as the maintenance of sterility of those parts of the equipment being used to cut, pick up or transfer explants. Improved systems must be developed which cater for these requirements, otherwise the labour components will be excessive and cost effective plant production could be nullified. The automated systems must be sterile and free of contaminants, and screening of tissues for bacteria on selected media for specific contaminants is worthwhile (AITKEN-CHRISTIE *et al.* 1995).

1.3.2. Growth and development

Plant genes determine the maximum potential for growth and developmental rates of cultures, however their actual rates are limited by their surrounding micro-environment. Systematic comprehension and control of the micro-environment is therefore required to achieve the full genetic potential of cultures and to establish a procedure that will make cultures exhibit their hereditary characteristics in a highly efficient and stable way (FUJIWARA & KOZAI, 1995). Environmental control gives tissue culture a distinct advantage for manipulating growth and development of plants. Environmental control of the *in vitro* system can be utilized to achieve good plant quality and high numbers for production (KOZAI & SMITH, 1995). The culture

environment is the result of the interaction between the plant medium, the culture container and the environment of the culture room. All of these interactions have an influence on a tissue culture system (DEBERGH & READ, 1991). Promotion or restriction of growth and development can be achieved by manipulation of the culture environment (KOZAI & SMITH, 1995). Plants grown *in vitro* are more sensitive to environmental parameters and less tolerant of change than those grown *ex vitro*. Thus, precise environmental control in plant tissue culture is critical.

Growth implies an increase in dry weight, fresh weight, leaf area, elongation of cells or organs and other aspects such as embryogenesis, organogenesis, branching, flowering, leaf unfolding, tuberization, and bulb formation. The *in vitro* environment affects the morphology of the plants. Some of the desired morphological characteristics of micropropagated plantlets at transplanting stage are: relatively large leaf area with an appropriate shoot/root weight ratio; a short inter-node length and shoot/plant height; and high resistance of leaves to water stress (KOZAI & SMITH, 1995). Conventional *in vitro* cultured plants tend to have little epicuticular wax formation, stomatal malfunction, a low chlorophyll content, fewer stomata on the leaves, poorly-structured spongy and palisade tissues and vascular systems, a low photosynthetic capacity, and incomplete rooting or few secondary roots. These are often undesirable characteristics and can be improved by proper environmental control (KOZAI & SMITH, 1995).

By understanding the inter-relationship between physical and chemical factors and by the ability to alter these cultural factors it is possible to: control growth and development, morphological and physiological characteristics of the plant; and reduce energy consumption (labour and use of supplies) in plant tissue culture (KOZAI & SMITH, 1995).

1.3.2.1. Physical environmental factors

The variables related to the culture vessel (size, shape, closure) or the medium phase (gelling agents, liquid medium or physical supports) can modify *in vitro* plant behavior, often more predictably and cost effectively than chemical additives to the medium. Variations in morphology and developmental stages of plants *in vitro* can be reduced, to some extent, by proper environmental control using well-designed culture vessels in a well-designed culture

room (HAYASHI, FUJITA, KITAYA & KOZAI, 1992). Physical environmental factors including: head space; growth room temperature; applying vessel bottom cooling to influence internal head space humidity; incident light at the culture surface; air movement; physically moving culture vessels to alternative growth room conditions; physical boundaries of the culture vessel; and physical characteristics of culture medium are predetermined and can be maintained as a constant, or varied during the culture growth cycle (HAYASHI *et al.* 1992). Establishing a method for effective control of the micro-environment should provide high production efficiency and improve product quality, thereby considerably expanding the application of plant tissue culture (FUJIWARA & KOZAI, 1995).

A. Head space, vessel type and vessel closure

Headspace (environmental variables of high relative humidity, unfavourable gaseous composition and little air movement) can be changed via environmental control by altering the type and size of the vessels, vessel closures and forced introduction of sterile humidified gases (McCLELLAND & SMITH, 1990; SMITH & McCLELLAND, 1991; KUBOTA & KOZAI, 1992; TANAKA, FUJIWARA & KOZAI, 1992). These factors are important as they have an influence on the other physical (gases, temperature and light) and chemical parameters. Manipulation by altering the physical environment has resulted in cultured plants with enhanced growth and superior ability to survive *ex vitro* as a transplant (KOZAI & SMITH, 1995).

In tissue culture, preventative measures, which can lead to poor aeration, are taken to protect cultures from contamination, (JACKSON, ABBOTT, BELCHER, HALL, BUTLER & CAMERON, 1991). The vessel closures regulate the degree to which physico-chemical factors in the growth room impact on the micro-environment (SMITH & SPOMER, 1995). The type of closure or vessel sealing material has been shown to affect gas composition in culture vessels, shoot multiplication, morphogenesis of plants, production of secondary metabolites, shoot quality, growth of woody shoots *in vitro* and occurrence of hyperhydricity. The type of vessels and closures used also influence temperatures. Vessels and closures exert significant influence on the headspace humidity and the headspace gaseous composition. Tightness of closure of vessels has also been associated with changes in culture responses (SMITH & SPOMER, 1995). In general loose-fitting closures were found to be better than tighter ones for improving growth and morphogenesis of cultures and for overcoming hyperhydricity (JACKSON *et al.* 1991;

FUJIWARA & KOZAI, 1995). The effect of improving growth can be explained in terms of the number of air exchanges per hour in the vessels. The number of air exchanges per hour, of culture vessels, can be increased by the incorporation of a gas-permeable micro-porous polypropylene film into closure or by increasing the airflow in the culture room (IBARAKI, IIDA & KURATA, 1992). Increasing the number of air exchanges per hour of a culture vessel effectively dilutes the concentration of toxic gases and supplies beneficial gases into the vessel (FUJIWARA & KOZAI, 1995).

The type of vessel selected for use is important. Light transmission, isolation from water loss and contaminants, allowance for gas exchange and growing area are all important considerations when choosing the vessels to use. These must also be autoclave resistant, easy to handle and transport, and washable (McCLELLAND & SMITH, 1990). McCLELLAND & SMITH (1990) found that woody plant explants produced denser shoot cultures when grown in large vessels and that the internal volume ratios of the vessels regulate the growth habits of *in vitro* plants. The quality of individual micro-shoots has been found to improve in larger vessels (size tested: 60 ml glass tubes; 200 ml baby food jars; 350ml polypropylene GA7 vessels). Shoot length was enhanced in many species, and the size leaves increased with vessel size. Rooting in the larger vessels was also improved (McCLELLAND & SMITH, 1990). MACKAY & KITTO (1988) found that culture vessel size had an effect on proliferation of axillary shoots. The effect of a larger air volume in larger vessels on the growth and development of cultures was beneficial because a preferable gas concentration and ratio of carbon dioxide, oxygen and ethylene was found in the vessels (FUJIWARA & KOZAI, 1995).

Relative humidity is influenced by the degree of closure tightness, which regulates the exchange between headspace air and the outside culture room atmosphere. Relative humidity above a gel-solidified nutrient medium surrounded by the vessel's walls and closure surfaces has been assumed to be very high *in vitro* approaching 95-100 % (SMITH & SPOMER, 1995). Vessels have been designed to increase headspace gaseous compositions.

Improved shoot morphology and growth have been achieved with the use of gas permeable membranes and micro-porous membranes. Selectively permeable membranes reduce headspace relative humidity and improve stomatal development and wax deposition on leaves.

Photoautotrophic *in vitro* systems or forced ventilation systems enhance carbon dioxide enrichment. By increasing the carbon dioxide, simultaneously increasing the irradiance at the plant surface and eliminating the typical sucrose in the culture medium, *in vitro* plants have been stimulated to productively photosynthesize (SMITH & SPOMER, 1995).

B. Gases

Oxygen and carbon dioxide are principal substrates or products of aerobic respiration and photosynthesis and can affect the most basic life-sustaining metabolic pathways of plant cells. Ethylene, in contrast, is a plant growth regulator capable of influencing developmental processes such as cell expansion, senescence and differentiation at relatively small concentrations (0.01-10 ppm (v/v)) (JACKSON, 2002). Without adequate aeration, plants suffer from a reduced influx of oxygen, while photosynthetic tissues can be deprived of the external carbon dioxide needed for the generation of dry mass (JACKSON *et al.* 1991).

In conventional tissue culture, carbon dioxide concentration in the airtight vessel is often as low as 100 ppm during exposure to light and the plantlets cannot develop a positive carbon balance (KOZAI, 1988). The micro-environment is dependent on the mass and energy exchange processes. Concentrations and the ability for diffusion to occur in the culture vessels are important factors of gaseous micro-environments. The differences in gas concentration and diffusion ability are due mainly to the small size of the culture vessel and minimal gas exchange between the inside and the outside of the vessel. Gas concentration in a vessel depends on the gas exchange rate of cultures and medium in the vessel and the physical properties of the vessel (FUJIWARA & KOZAI, 1995).

Increasing carbon dioxide concentrations to a certain level is known to enhance photosynthesis for many greenhouse and field plants. Photoautotrophic micropropagation is a method of growing cultures without adding any carbon sources or organic salts to the medium and of promoting the photosynthesis of chlorophyllous shoots by increasing both carbon dioxide concentration and photosynthetic photon flux density inside the vessel up to the appropriate levels. DE RIEK, VAN CLEEMPUT & DEBERGH (1991) devised a carbon flow scheme for cultures. This scheme details the complex nature of carbon relations to cultures, which result from the fact that cultures utilize sugars in the medium and carbon dioxide in the headspace of

the culture vessel. ZOBAYED, ARMSTRONG & ARMSTRONG (1999) found that in the light period, carbon dioxide depletion occurred in the headspace of the sealed vessels. In that work, the carbon dioxide concentration increased with the increasing efficiency of the ventilation. Further, no ethylene accumulation was noticed in the headspace of the culture vessels when humidity-induced through-flow ventilation was applied. However, high ethylene accumulation occurred in sealed vessels.

The headspace air of a vessel is the source of oxygen supply for respiration of the plants. Higher concentrations of oxygen in the headspace produce higher survival rates. Oxygen concentrations show a cyclic change with the lighting cycle. During the dark phase there is a decrease in oxygen with an almost equal increase in carbon dioxide concentration, but this is dependent on the type of carbon fixation of the system (FUJIWARA & KOZAI, 1995).

The success of plant tissue culture depends on the external control of morphogenesis, mainly by plant growth regulators. For most plant growth regulators the control is more or less deliberate but this is often not the case for the only gaseous plant growth regulator, ethylene (MATTHYS, GIELIS & DEBERGH, 1995). According to these authors, ethylene is known to influence different facets of tissue culture e.g. bud development, embryogenesis, and rhizogenesis, anther culture and flowering *in vitro*. Further, the ethylene effect is not clear-cut and can be stimulatory or inhibitory for the process considered. Ethylene is formed when plants are subject to stress conditions, and in culture containers ethylene concentrations are dependent on both external conditions (illumination, temperature, atmospheric pressure, air pollution) and internal conditions (plant, container type and closure, headspace, volume, medium, temperature, pressure etc.) as well as their interactions. By choosing proper experimental conditions (culture container, gelling agents, illumination) it is possible to avoid its production to some extent (MATTHYS *et al.* 1995).

The concentration of ethylene in a relatively airtight culture vessel increases gradually with time. Most ethylene problems result when the concentrations become too high in the culture vessel (ethylene is considered to be released by the plants only). Different types of tissue accumulate different levels of ethylene thereby affecting the growth (KUMAR, JOY (IV) & THORPE, 1989). JACKSON *et al.* (1991) found a value (t_{50}) with which to compare ethylene

concentration in different vessels. Ethylene was injected into the vessels and its rate of loss was monitored. The time in hours for half the ethylene to be lost was calculated (t_{50}). They found that ethylene and carbon dioxide accumulated in a sealed container. Some plants were affected by this accumulation and had smaller leaf and shoot sizes. KUMAR, REID & THORPE (1987) indicated that both ethylene and carbon dioxide build up during the first 10-15 days of culture and promote morphogenesis. However, excessive accumulation after the initiation of buds then caused dedifferentiation.

Ethylene in the vessel has to be released to the outside of the vessel once accumulated. Increasing the number of air exchanges of the vessel is the best way to solve the problem. Forced ventilation systems are employed to enhance photoautotrophic growth of cultures or to allow air exchange to reduce the ethylene concentrations in the vessel. If there is no forced ventilation, ethylene has to be released by diffusion. The amount of diffusion depends on the culture conditions such as ventilation, closure of vessels, headspace and medium agitation (FUJIWARA & KOZAI, 1995).

C. Light and temperature

Light has a major influence on the growth, development and morphogenesis of plants. In an *in vitro* environment where conditions are manipulated to optimize a given response, careful consideration should be given to light quantity, quality and intensity as well as the photoperiod (ELLIS & WEBB, 1993). By controlling the temperature, photosynthetic photon flux density (light intensity) and red/far red ratio of the light source, the shoot length of *in vitro* plants can be controlled (HAYASHI *et al.*, 1992). HAYASHI *et al.* (1992) suggested that changing the position of the light source to the side, rather than overhead, enhanced growth and morphological development in potato plantlets grown *in vitro*. Plants respond to light in three general ways: photoperiodism - the response to duration and timing of day and night; phototropism - growth based on direction of the light source; photomorphogenesis - the influence of light on the development of the plant. The level of response can depend on the way in which light is presented to the plant, i.e. the light quality, intensity and duration of exposure (ELLIS & WEBB, 1993).

Light flux density is regarded as one of the most important parameters, especially when it involves the photosynthesis of cultures. It has been shown that relatively high light flux densities promote the photosynthetic rates of the cultures. Light flux density in culture vessels is affected by the type and number of the light sources; the material and shape of culture vessels; the position of the vessel on the culture shelf; the position of the light source; and the optical characteristics of the shelf (FUJIWARA & KOZAI, 1995). SVENSSON (2000) reported that the photon flux levels affect multiplication and subsequent rooting in *Aristolochia manchuriensis*. It was found that a prolonged period of a high flux increased multiplication but affected subsequent rooting negatively. It is evident that the type of light used can influence the growth of plants. BACH, MALIK, PTAK & KEDRA (2000) tested different coloured lights to determine the effects these had on plant growth. Red and yellow promoted embryogenic callus and somatic maturation. Blue and ultra-violet light stimulated development of somatic embryos but inhibited maturation. STASINOPOULOS & HANGARTER (1990) stated that the spectral transmissions of culture vessel materials differ from each other. Polycarbonate, glass and polystyrene culture vessels do not transmit light wavelength shorter than 390, 209 and 300nm respectively. When selecting a light source for a culture room the spectral distribution of radiation from the light source as well as the incident light flux density on the culture shelf must be taken into consideration. The lighting cycle is of importance in carbon dioxide uptake. FUJIWARA & KOZAI, (1995) reported that carbon dioxide uptake by plantlets *in vitro* increased with shortening the lighting cycle and that the relative humidity in culture vessels is affected by the lighting cycle.

Temperature differences are caused directly or indirectly by incident light from lamps and heat exchange between the outer surface of the vessel and air outside the vessels. The air temperature in culture vessels is considered to be almost the same as that outside the vessel or in the culture room during almost all the dark period. The air temperature difference between the inside and outside of a culture vessel increases with increased irradiance. The highest air temperature inside the vessel is at the surface of the culture medium where the radiation is mostly absorbed and converted into heat to raise the temperature. The air temperature difference between the inside and outside of a culture vessel is also dependent upon the vessel geometry, optical transmission of the vessel material, airflow speed around the vessel, and the number of air exchanges of the vessel. Micropropagated shoots/plantlets tend to be tall and thin due to

characteristics of the micro-environment in culture vessels. Tall and thin shoots/plantlets are prone to lodge when they are transplanted in the greenhouse or in the field. Therefore short and thick shoots/plantlets are desirable when they are used as transplants (FUJIWARA & KOZAI, 1995).

D. Gelling agents, water micro-environment and hyperhydricity

Gelling agents are complex polysaccharides which, when dissolved in water or an ionic solution, form cross-links between the macromolecules to give a semi-solid consistency to the solution (JONES, 1993). They are frequently employed in plant tissue culture to impart viscosity or semi-solid consistency to liquid media, and contribute to the status of the micro-environment. Gels alter water availability to the growing plant by affecting the water relations in the culture vessel and usually contribute contaminants in the form of extra minerals to the chemical environment. Gelling agents decrease the availability of water and dissolved substance to the explants. A reduced gel concentration will result in an increase in water availability and mobility of ions in the water phase of the medium. Increased gel concentrations will decrease water availability to the explants (SMITH & SPOMER, 1995).

In vitro plants are sensitive to differences in media and gelling agent type and concentration. The gel strength and conductivity of the gel must be considered, as it will seriously change the performance of explants in culture. JONES & PETOLINO (1988) pointed out that the presence or absence of agar influenced the different stages of embryo culture of *Triticum aestivum* L. The gelling agent controls nutrient availability, although ideally it should be chemically inert. The gel governs how efficiently nutrient molecules diffuse through the medium. The matric potential of the agar affects the ability of a plant to take up nutrients from the semi-solid medium. Ions bind to the gel surfaces, and diffusion is hampered when gel pore sizes are small (SMITH & SPOMER, 1995). Both the brand and concentration of agar affect the chemical and physical characteristics of the culture medium. Impurities introduced with agar are responsible for significant differences in the concentration of elements in comparable media (DEBERGH, 1983). TANIMOTO & ISHIOKA (1991) and CASSELLS & COLLINS (2000) tested different gelling agents and found the occurrence of inhibition of growth on different types of agar. Differences between gelling agents in terms of gel binding sites are likely to influence nutrient availability to explants. It is apparent that different physiological responses are a reflection of

different water and nutrient availability in the different media. BERUTO, CURIR & DEBERGH (1999) tested three different gels and found that one caused hyperhydricity, another enhanced fresh and dry weight while the third caused stomatal deformities on *Ranunculus.* Gelrite promoted shoot growth in walnut whereas agar inhibited growth (BARBAS, JAY-ALLEMAND, DOUMAS, CHAILLOU & CORNU, 1993). Gelrite also has been reported to cause less release of ethylene from the plants than agar (MATTHYS *et al. 1*995). Not only does the type of gel influence growth but also the concentration has an effect on growth. VON ARNOLD & ERIKSSON (1984) reported that an increase in agar concentration decreased hyperhydricity, however a reduction of shoot growth and rooting potential occurred. BORNMAN & VOGELMANN (1984) found an inverse correlation between the N^6-benzyladenine accumulation and the degree of gel stiffness and that greater numbers of adventitious buds were induced at low to medium levels of rigidity.

Water is the main constituent of culture media. Its free-energy status and availability in media are directly associated with water transfer which exerts influences on almost all important physiological activities, such as nutrient absorption and transpiration of cultures. High concentrations of agar in the medium results in lower relative humidity and consequently promote acclimatization of plants (FUJIWARA & KOZAI, 1995). Although the micelle structure of a gel creates aeration pore spaces in the rooting zone of an *in vitro* cultured plant, roots generated in a semi-solidified medium exhibit an abnormal morphology compared with roots produced in a soilless greenhouse mix. Immature root vascular systems, irregular intercellular gaps and larger, hypertrophied individual cells *in vitro* are a consequence of the poor aeration and saturated conditions in a gel matrix. Roots are frequently unable to form *in vitro* in static liquid medium. However the use of mist systems significantly enhances root growth coincident with increased aeration (SMITH & SPOMER, 1995). Effects of relative humidity or water vapor deficit in the vessel can cause physiological, morphological or anatomical changes in shoots/plantlets (leaf wax deposition, stomatal function, leaf resistance to water vapor transfer; growth and wilting after transplanting). High relative humidity in culture vessels resulted in physiological and morphological disorders of cultures. Reduction in relative humidity in the vessel to an appropriate level may provide ways to improve the physiological and morphological characteristics of cultures as well as to produce shoots/plantlets more able to

withstand water stress after transplanting from *in vitro* to *ex vitro* conditions (FUJIWARA & KOZAI, 1995).

Primarily as a consequence of enhanced water availability, liquid medium systems have often yielded superior shoot culture growth as compared with agar-solidified systems. Gel free culture systems have involved bubbling or agitated/aerated bioreactor culture, agitated suspensions, static thin layer liquid film systems, mist application of liquid medium, use of rafts or filter paper bridges, various physical supports and double phase liquid systems. Superior performance on liquid versus semi-solid media has been ascribed to enhanced water availability, removal of agar impurities, and reduction of mechanical impedance. Liquid systems very frequently yield faster, more prolific growth. Preventative techniques to alleviate hyperhydricity, yet capture the benefits of liquid media systems, include partial immersion of plants to ensure aeration, use of dual phase (overlay) culture systems, use of neutral absorbent substances, direct oxygenation of the medium and control of headspace humidity. Liquid culture systems are better from a commercial production standpoint as media can be changed easily, and sterilization and cleaning of media from vessels is greatly simplified (SMITH & SPOMER, 1995).

Many of the plant tissue culture quality changes associated with the medium phase are a consequence of hyperhydricity. This adverse plant tissue condition is very strongly linked to the medium phase and occurs in particular when a medium has insufficient gel strength. Gelling agents can help prevent hyperhydricity. JONES (1993) stated that shoot cultures of many species might become slow-growing with tightly rolled transluscent leaves, a process, which has been described as hyperhydricity. In apple it was found that by modifying the brand and concentration of agar and type of carbon and the concentration of ammonium ions hyperhydricity could be eliminated. KEVERS, COUMANS, COUMANS-GILLÈS & GASPAR (1984) hypothesized that hyperhydricity results from a burst of ethylene controlled by the peroxidase-IAA-oxidase system. DEBERGH, HARBAOUI & LEMUER (1981) discovered that the only way they could eliminate hyperhydricity in artichoke (*Cynara scolymus*) was by raising the agar concentration. MAJADA (1998) used gas permeable caps and controlled the ventilation rates to eliminate hyperhydricity. PHAN (1991) observed that it was neither the physical state nor ethylene that were the causal agents for hyperhydricity, but that cytokinins induced the abnormality by promoting excessive cell divisions at the expense of cell differentiation. ZIV

(1991) reviewed the culture conditions on morphology and physiological changes related to hyperhydricity of micropropagated plants. The review discussed the various metabolic and physiological disorders and the effects that occurred on *in vitro* produced plants by the culture conditions (physical and chemical).

It is essential to be aware of the potential changes that can be imposed by vessels, gel strength etc. When control can be achieved by manipulation of physical factors rather than by chemical agents, the benefits, including reduced environmental pollution, lower cost, and a higher degree of management, are manifold (FUJIWARA & KOZAI, 1995).

1.3.2.2. Chemical micro-environmental factors

The *in vitro* chemical environment can be changed by changing the physical environment (sub-culturing a plant, opening a vessel lid to change gaseous composition, gels - either different types and different concentrations) or by changing the chemical composition (growth regulators, sugar, and nutrient composition) (KOZAI & SMITH, 1995). Plant *in vitro* culture media are composed of several groups of components, mineral ions, growth regulators, sucrose and various other organic substances with or without the use of a gelling agent. The composition of medium used for a particular plant species or culture type is usually developed by empirical manipulation of ion combinations of formulas and these have evolved over time (WILLIAMS, 1995). Different responses *in vitro* may be brought about by different mineral salt formulations and different concentrations of the minerals (e.g. half or double strength media). Total mineral uptake and plant growth tend to be closely correlated (KIRSCHBAUM, 1991). GAMBORG & SHYLUK (1981) have reported on the nutrition and media that are required, together with the growth characteristics, for different types of cultures (callus, cell, organ, meristem, and protoplast). GEORGE, PUTTOCK & GEORGE (1987, 1988) reviewed many different formulations that have been used by different people for different plant types and culture types. Those reviews revealed how media adaptations have evolved to enhance growth and morphology of many plant species over the years. Different genotypes of plants require different media formulations. BERGMANN & STOMP (1992) indicated that there are significant differences in shoot production of *Pinus ocarpa* provenances on a specific medium. This suggested that although the plants may be genetically related, there is specificity in the medium requirement.

The mineral ion component of the medium must provide macro-elements and micro-elements normally required by the plants (WILLIAMS, 1995). The nutrients must be available in a suitable soluble form and in proportion to avoid deficiencies or unbalanced uptake. The type and quantity of minerals supplied can be controlled at the beginning of a new culture cycle. However, the physical and chemical changes taking place in the medium and the interaction with the plants cannot be controlled. The medium and plants are in a state of flux within the cycles and from one subculture to the next (WILLIAMS, 1995). Ions may not remain readily available to the plant and the relative concentrations in the medium change due to differential uptake by the plant. The constituents of the semi-solid medium are not necessarily evenly distributed through the medium or equally available to the plant (KOZAI & SMITH, 1995).

Minerals present in the medium are used by the plants as building blocks for the synthesis of organic molecules, or as catalysts in enzymatic reactions. The ions of the dissolved salts play an important role in the transportation of ionized molecules by the plant, in the osmotic regulation and in maintaining the electrochemical potential of the plants (DUCHEFA CATALOGUE, 1998-1999). Cultures vary widely in their response to higher overall mineral supply. Some woody species grow better on media with lower ionic strength. Supra-optimal concentrations of mineral ions such as chlorine, and ammonium may cause hyperhydricity. Omission of nitrates, ammonium, phosphates, and potassium may inhibit growth. Further, the optimum mineral requirements can vary between the stages of culture growth (WILLIAMS, 1995). With *Eucalyptus*, KIRSCHBAUM (1991) found that growth of seedlings was proportional to the internal concentration of nutrients, but most media for micropropagation of *Eucalyptus* via shoot regeneration and multiplication include a gelling agent. Liquid media have not been extensively used for regeneration and multiplication of shoots until recently. With the current developments towards autotrophic *in vitro* cultures, the optimum requirements for minerals in the medium need to be re-examined. (WILLIAMS, 1995).

A. Macro-elements

Essential elements required by the plant in large amounts are termed macro-elements e.g., calcium, magnesium, nitrogen, phosphorous, potassium and sulphur (DODDS & ROBERTS, 1985).

Nitrogen (N) is added to the culture medium in the largest concentration. Nitrogen is either present as nitrate or ammonium and, as a component of proteins and nucleic acids, it is therefore of prime importance for all plant growth. It is also a structural component of the cell wall (DELL, 1996). The relative supply and uptake can affect culture growth indirectly by its effect on the pH of the medium. The ratio of ammonium and nitrates can influence morphogenesis (WILLIAMS, 1995).

Potassium (K) which is second to N in its abundance, is readily absorbed by plant tissues. Potassium is a monovalent cation with a high mobility in the plant at cellular levels and in the transport over longer distances in the xylem and phloem. Potassium salts have an important function in the osmotic regulation of the cell and in the stabilization of pH (GEORGE *et al.* 1988; DELL, 1996; DUCHEFA CATALOGUE, 1998-1999). The level of K required for maximum growth varies widely between species, with minimum levels for some species being toxic to others. Tissue levels of K are a reflection of the supply rather than demand by the plant. It is rarely a problem *in vitro* (WILLIAMS, 1995).

Phosphorous (P) may often be the limiting mineral in plant cultures because of its relatively poor availability and particularly under autotrophic conditions where its utilization in phosphorylation is high. The highly energetic pyrophosphate bond of phosphorus when bound to another P atom, as in ATP, is very important for the energy metabolism of the cell (GEORGE *et al.* 1988; DUCHEFA CATALOGUE, 1998-1999). It is a structural element of nucleic acids, phospho-lipids and phospho-proteins (DELL, 1996).

The supply of sulphur (S) is usually adequate with agar often containing significant amounts. With the use of MURASHIGE & SKOOG (1962) medium, sulphur can be limited (WILLIAMS, 1995). Sulphate has to be reduced before it can be used for the synthesis of reduced S containing compounds like amino acids, proteins and enzymes (DELL, 1996 & DUCHEFA CATALOGUE, 1998-1999).

Calcium (Ca) plays an integral role in the control of cell wall synthesis and maintenance of membrane integrity (DELL, 1996). An adequate supply of Ca is essential for plant growth but high concentrations of Ca inhibit cell extensions while promoting secondary wall deposition of

callose. Cytoplasmic Ca is involved in the regulation of plant growth regulator responses. Calcium mediates in its responses to environmental factors such as light and temperature. Plants grown on higher Ca have more open stomata. Shoot tip necrosis occurs as a symptom of Ca deficiency, which is often due to poor distribution and transport of Ca rather than a deficiency. There are strong interactions between calcium, magnesium and boron with some compensation between them, therefore the correct tissue levels of Ca need careful interpretation. A pre-emptive regulatory role in morphogenesis may occur due to Ca (GEORGE *et al.* 1988; WILLIAMS, 1995; DUCHEFA CATALOGUE, 1998-1999).

Magnesium (Mg) is a component of chlorophyll and co-factor for many enzyme reactions. It may substitute for Ca in some non-specific roles. Where Ca uptake is limited, the supply of Mg may be important. Magnesium ions are involved in the regulation of the intracellular pH and the correct cation balance. Magnesium uptake is not usually limited except at low pH (WILLIAMS, 1995).

B. Micro-elements

In addition to macro-elements, plant cells require traces of certain micro-elements (boron (B), chlorine (Cl), iron (Fe), cobalt (Co), copper (Cu), manganese (Mn), molybdenum (Mo) and zinc (Zn)). These are needed in very small quantities by the plants. They are essential as catalysts for many biochemical processes. It is generally assumed that sufficient quantities may be obtained as impurities in a culture medium to enable normal plant growth. It is difficult to discern their precise effect on culture growth but deficiency can have adverse effects on growth and development (DODDS & ROBERTS, 1985; GEORGE *et al.* 1988; DUCHEFA CATALOGUE, 1998-1999). General symptoms of micronutrient deficiencies include leaf chlorosis (Fe, Zn, Mn), reduced lignification (Cu, Fe), rosetting (Zn, Mn) and shoot tip necrosis (B). Other elements such as cobalt (Co) and nickel (Ni) are not essential but may indirectly influence culture growth, e.g. Ni and Co can inhibit ethylene synthesis. Chlorine is taken up as Cl^- and is mobile in the plant, and it functions in osmoregulation and compensates for other ionic charges. Micronutrients interact with other chemical processes (WILLIAMS, 1995). For example, TRINDADE, FERREIRA & PAIS (1990) reported that boron (B) and aluminum (Al) interact with auxins in the initiation of adventitious roots on *Eucalyptus*. Iron is usually more critical

than other micronutrients because of the larger levels required and the solubility problems. Iron forms iron chelates in the cells (WILLIAMS, 1995).

C. Vitamins

Vitamins are beneficial as they are used for many biochemical reactions. Thiamine and myo-inositol (although this is a sugar alcohol it is used as a vitamin) are the vitamins most frequently included in plant culture media along with pyridoxine and calcium-pantothenate. Vitamins use myo-inositol as a carrier molecule for transport across plant and cellular membranes and it is for this reason that the carbohydrate is included in tissue culture media. Thiamine and myo-inositol are involved in cell biosynthesis and metabolism. Growth may also be improved by the inclusion of other non-essential vitamins, particularly nicotinic acid or pyridoxine. The requirement for additional vitamins may develop over several culture cycles presumably as the tissue uses the endogenous supply present at the time of sub culturing (GEORGE *et al.* 1988; WILLIAMS, 1995 & DUCHEFA CATALOGUE, 1998-1999).

D. Plant growth regulators

Plant growth regulators are usually the key to control of plant growth and development in culture. The requirements of growth regulators by plant cultures are normally auxins (stimulate shoot cell elongation) and cytokinins (promote cell division) (DODDS & ROBERTS, 1985). A deliberate change in growth regulators added to a tissue culture medium can cause a dramatic response in cultured plants. Due to this cause/effect relationship, growth regulator treatments should be carefully designed based on the anticipated consequences to *in vitro* performance (WILLIAMS, 1995). Growth regulators have a profound influence in regulating organized development *in vitro* (THORPE & KUMAR, 1993). The influence of growth regulators on differentiation has long been demonstrated. Their exact role in this process however is not clear-cut because each class of growth regulator elicits a wide range of responses in different parts of different plants. The response varies qualitatively depending not only on the concentrations or ratios of the regulatory chemicals present, but also on the physiological status of the tissues or plant. PREECE (1995) in his review stated that if the optimization of nutrient salts in the medium could be achieved, it would be possible to reduce the concentrations of plant growth regulators. It is however, the balance of cytokinins and auxins supplied in the medium that play a pivotal role in the type of growth (THORPE & KUMAR, 1993; WILLIAMS, 1995).

VILLALOBOS, LEUNG & THORPE (1984) discovered that not only are the relationships within the growth regulators important, but so are the relationships with other environmental factors. Using *radiata* pine they showed that there were morphogenetic interactions of light and cytokinin in shoot formation. Cytokinin is directly involved in the induction of shoot initiation and both light and cytokinin are required for the development of meristematic tissue and subsequent shoot formation.

E. Carbohydrates

There is a need for the supply of carbohydrate in the medium as a source of energy and carbon substrate. The most common carbohydrate source is sucrose but other sugars such as fructose, glucose and sorbitol have been used for some species. The actual requirement for carbohydrate depends on the environmental conditions, particularly light intensity and the carbon dioxide supply. The carbohydrate requirement also varies between plant genotypes, e.g. variations amongst *Eucalyptus* clones (DAMIANO, CURIR & COSMI, 1987). Sucrose between two to six percent in the medium was favoured for *Eucalyptus* root development. Other concentrations were found to be detrimental to the explant cultures (CHENG, PETERSON & MITCHELL, 1992). THOMPSON & THORPE (1987) and DESJARDINS, HDIDER & DE RIEK (1995) have extensively reviewed the plant responses to carbohydrates supplied in the medium. The osmotic contribution of carbohydrate in the medium is important because it affects the availability of water and hence mineral uptake and plant growth. Changing the level of sucrose in the medium may affect the pattern of morphological development (WILLIAMS, 1995). In *Picea abies* it was found that bud formation could occur on medium without sucrose but sucrose was required for further development of meristematic centers (VON ARNOLD, 1987). KOZAI (1988) stated that plantlets *in vitro* have been considered to have little photosynthetic ability, so sugar has to be provided as a carbon source in the culture medium. *In vitro* plantlets do have photosynthetic ability and can develop autotrophy, provided that physical environmental factors such as carbon dioxide and light in the culture vessel are properly controlled, in which case no sugar for growth is required in the medium. Raising the carbon dioxide levels and maintaining cultures under high light intensity facilitates autotrophic growth, which then allows the reduction or elimination of sugar from the medium (THOMPSON & THORPE, 1987; DESJARDINS *et al.* 1995; WILLIAMS, 1995).

F. pH of the media

The pattern and occurrence of morphogenesis *in vitro* may be regulated by the pH of the medium (WILLIAMS, 1995). The pH of the medium is important for the gels to solidify and it also affects the nutrient uptake and solubility of ions, which in turn affects the growth and morphology of the plants. Plants grow between a pH of four to seven if nutrients do not become a limiting factor (BUGBEE, 1996). Availability of manganese, copper, zinc and iron is reduced at a higher pH, and there is a decrease in the availability of phosphorous, potassium, calcium and magnesium at lower pH (COOPER, 1996). The uptake of different cations and anions by the plants causes shifts in the pH (ERREBHI & WILCOX, 1990). OWEN, WENGERD & MILLER (1991) recorded that gelling agents and activated charcoal increased the post-autoclave pH. The pH in the medium should be adjusted after the addition of the gelling agent and prior to autoclaving to alleviate pH changes.

1.3.3. Rooting and acclimatization

Successful results for *in vitro* cloning have been reported for many plant species, but it is the rooting and acclimatization which are the critical and limiting steps (ZIV, 1995). The survival of plants after transfer to soil is of vital importance as poor survival decreases the propagation efficiency and increases the production costs (DESJARDINS *et al.* 1995). According to KOZAI, (1988) and DESJARDINS *et al.* (1995) the problems of poor survival originate from the following or combinations of the following:

- poor control of water loss caused by high relative humidity found *in vitro*
- poor photosynthetic rate of plantlets which has been attributed to the presence of sugar in the medium
- light which is too low
- inadequate carbon dioxide supply
- incomplete autotrophy
- high transpiration rate due to a thin cuticular layer and abnormal stomata (reduced deposits of epicuticular waxes and the inability of the stomata to function after removal of the plants from culture, are the major causes for water loss and desiccation)
- incomplete rooting (lack of functional vascular tissue with poor connection between the shoot and the root system often restricts water uptake)

- physiological disorders
- hyperhydricity

Roots develop either directly from the stem or indirectly via wound tissue (GRÖNROOS & VON ARNOLD, 1987). Roots are indispensable organs for a positive water balance. They can be induced both *in vitro* and *ex vitro* by auxins, but *ex vitro* produced root systems are far better adapted to survive acclimatization. Auxins are used in conventional micropropagation to induce formation of root primordia (*in vitro*) and to promote extensive rooting *ex vitro*. Many different combinations of plant growth regulators are used to induce rooting for the different species of plants. ABDULLAH, GRACE & YEOMAN (1989) stated that cytokinin and auxin and the interactions between them affected the quantity and quality of induced roots on *Calabrian* pine. CHENG *et al.* (1992) tried different concentrations of IBA and NAA in the media and found that a combination of these two hormones (2.5 μM IBA and 2.5 μM NAA) was suitable for rooting in *Eucalyptus*.

Control of the micro-environment in the culture vessel can improve acclimatization procedures, enhance plant growth and increase plant survival *ex vitro*. Most shoots can be induced to initiate roots in culture, however the immediate contribution of properly developed roots to plant survival and low production costs are inconclusive and depends on the technique used and the species (ZIV, 1995). During hardening, a defined physical environment with controlled light, gas exchange and relative humidity are prerequisites for plant acclimatization. PREECE & SUTTER, (1991), DONNELLY & TISDALL (1993), ZIV (1995) and DESJARDINS *et al.* (1995) reported extensively on the physiological aspect of the plants produced *in vitro* to obtain successful acclimatization. According to those authors, accelerated acclimatization can be achieved if the plants established *in vitro* developed a good shoot and root system. Modification of the *in vitro* production phases to more closely resemble *ex vitro* conditions will also contribute significantly to reduction in cost, resources, space and energy, and advance the achievement of economical micropropagation schemes. Further, the procedures employed in conventional *ex vitro* acclimatization (a gradual decrease in the relative humidity, removal of sugar, elevated irradiance and carbon dioxide) should be incorporated earlier in the production regime - during the preparation and hardening stages in culture. These modifications enhance the development of normal and adequate plant structure for efficient physiological functioning, improving shoot

and root quality and increasing the survival rates of the plants *ex vitro*. Cultured plant performance *ex vitro* varies greatly and at least during the first two to three days the ability of the plants to maintain a positive water balance is more pivotal than the photosynthetic performance. It must therefore be emphasized that *in vitro* acclimatization should provide a micro-environment to develop leaf and root structures that can withstand transpiration and support photosynthetic activity under stress conditions during the early phases of acclimatization *ex vitro*.

In conventional *in vitro* acclimatization practices the need for a gradual decrease in relative humidity, higher carbon dioxide and light levels and depleted medium is emphasized. Some of the methods employed to reduce humidity vary from the use of desiccants, uncapping of the culture vessels for up to one week prior to transplanting, bottom cooling to reduce the relative humidity in the headspace of the container, use of appropriate ventilation and culture lids with permeable membranes. The introduction of advanced techniques for acclimatization *in vitro* include several strategies: irradiance; relative humidity; carbon dioxide and other gases exchange - all aimed at producing photoautotrophic quality plants. HORGAN & HOLLAND (1989) introduced a pre-rooting treatment for *radiata* pine with high sucrose to enhance rooting. Plant photosynthetic performance is second in importance to the water balance during the first 24-48 hours after transplanting. According to AHUJA, (1993) the transition between the *in vitro* environment with almost 100 % relative humidity, to the *ex vitro* environment of field conditions with about 50 % relative humidity is critical for the survival of plantlets. It is therefore necessary to gradually lower the humidity from 100 % to field conditions.

The use of agar as a solidifying agent results in adventitious roots with poorly developed vascular connections, little to no secondary thickening (for woody plants), a loose cortical cell arrangement, pigmented cells, and several other features which interfere with successful *ex vitro* acclimatization. In woody species roots developed in agar are thick, have larger hypertrophied cortical cells and lack a secondary vascular system. As a result, only a percentage of the *in vitro* initiated roots may survive *ex vitro* acclimatization and, depending on species, the original roots may be replaced with new *ex vitro* root initials. Plant acclimatization in liquid medium, usually on some kind of support system, can provide a suitable micro-environment for the growth of root and shoot systems, eliminate the need for agar removal and decrease handling costs (ZIV, 1995). Liquid medium can be supplemented as a second phase on the agar layer or introduced and

removed automatically. Shoot elongation, rooting and overall enhanced growth can be achieved efficiently by a double layer technique introducing a liquid nutrient layer on top of the agar in herbaceous and woody plants (MAENE & DEBERGH, 1985). A liquified medium combined with a supportive system and a controlled micro-environment produced normal, good quality easy to handle plants. The importance of having a certain number of leaves and a good root system before transferring the plantlets from an *in vitro* to an *ex vitro* environment has been emphasized (VAN TELGEN, VAN MIL & KUNNEMAN, 1992; AHUJA, 1993). PINKER (2000) stated that there was an interaction of stem quality and rooting in *Prunus* and *Amelanchier* cultures. An improved quality of shoots prior to rooting led to increased rooting and acclimatization. The stem properties and rooting performance were affected considerably by the duration of the last subculture on multiplication medium. ZIV, (1995) and UOSUKAINEN, RANTALA, MANNINEN & VESTBERG, (2000) suggested that growth retardant (paclobutrazol or ancymidol) given at a suitable developmental stage and optimal level during acclimatization under photoautotrophic conditions could become promising bioregulators for *in vitro* plant quality, thus improving plant rootability.

The successful *ex vitro* acclimatization of micropropagated plants determines the quality of the end product and, in commercial production, the economic viability of the enterprise (DONNELLY & TISDALL, 1993). DAVIES & SANTAMARIA (2000) discussed a range of techniques available to assess the physiological competence of microplants, which could be used in future to assess photosynthetic ability of plants prior to rooting. Ensuring that the plants are more photautotrophic (by changing the physical and chemical environment) with better developed roots would enhance survival of tissue culture plants, thus lowering costs and making tissue culture more viable (ZOBAYED, AFREEN & KOZAI, 2000).

1.3.4. Problems with the use of *in vitro* culture systems

There are a few problems that arise when culturing plants *in vitro*. The inability to repeat results from one experiment to another and from batch to batch of plantlets, or somatic seedlings being produced are two such difficulties. These reduce the ability of the production facility to obtain consistent results and are an ongoing problem in conventional plant tissue culture. This is not unique to tissue culture and is commonly found in other vegetative propagation methods. It

occurs frequently in plants and is a function of their biology, physiology, biochemistry and adaptability to different environments. This unpredictability of plant growth and development also affects the repeatability of results from any automated system developed for handling plant tissues *in vitro*. Control over the uniformity and quality of plant growth may be obtained with manipulation of the physical and chemical micro-environments and this can positively affect the repeatability of results. Other approaches to produce a more uniform plant product have been to synchronize plant growth and to design and construct automated systems to cope with asynchronous growth (AITKEN-CHRISTIE *et al.* 1995).

1.3.5. Costs for conventional *in vitro* culture of plants

Environmental control is applied not only to influence plant growth *in vitro*, but also to allow practical control over operation economy (KOZAI & SMITH, 1995). Millions of plants are produced worldwide every year through micropropagation. However these methods are labour intensive and there has to be a good reason for choosing micropropagation (HEYERDAHL, OLSEN & HVOSLEF-EIDE, 1995). It has been widely recognized that application of micropropagation to plant production is at present, best restricted to crops with a high cost per unit. This is largely due to the high labour demand of dissection and transfer of plantlets to new culture vessels (KURATA, 1995). Tissue culture is frequently more expensive than other forms of propagation because, in addition to labour, it requires more specialized environmental control throughout the numerous stages of development. This has been a major constraint to the larger scale deployment of tissue culture plants (AITKEN-CHRISTIE *et al.* 1995). KOZAI (1988) stated that the main reasons which give rise to high production cost of *in vitro* plantlets are: the time taken for multiplications to occur; cost of preparation and acclimatization of the plantlets; and contamination losses at the multiplication and preparation stages.

Due to the increasing cost of labour in developed countries, conventional micropropagation systems have been challenged by high production costs and low gross profit. Only if the crop is so valuable in itself, or the product from micropropagation is of superior quality (therefore able to obtain a higher price), or traditional propagation is too costly, difficult or impossible is micropropagation chosen to solve the problem (HEYERDAHL *et al.* 1995). In general, most of the micropropagation companies in high labour cost areas have had to either automate their

system to reduce labour requirements, or move operations to a low labour cost area to reduce their production costs (CHU, 1995). Labour cost accounts for 60 to 70 % of the total production costs (KOZAI, 1988). The need for mechanization/automation of the micropropagation process has been claimed to reduce labour costs (KURATA, 1995). A relatively large culture vessel with an environmental control mechanism will be effective for labour saving if it does not give rise to an increase in microbial contamination. Therefore control of the *in vitro* environment in general and especially in photoautotrophic plant tissue culture, may contribute to savings in costs by reducing the loss of cultures *in vitro* due to microbial contamination and physiological /morphological disorders (KOZAI & SMITH, 1995).

Costs of basic components, sugar, agar and vessels are significant (KOZAI, 1988). Most supplies required for *in vitro* culture such as agar are neither re-used nor recycled. Furthermore, only some of the organic and inorganic nutrients added to the media are absorbed by the cultures *in vitro* during the culture period and the residual nutrients, including sugar, are discarded together with the gelling agent after culture. This current approach is not ecologically sound in the light of escalating environmental concerns and constraints. Minimum use and the recycling of nutrients, supporting materials and energy will become important in future tissue culture systems. In this regard the *in vitro* environment could be modified to allow extended culture cycles with refreshment of media via liquid overlay, to avoid simple repeated discards of media (AITKEN-CHRISTIE & DAVIES, 1988). MAENE & DEBERGH (1985) utilized this overlay system to save manual labour at the elongation and rooting stages. They found that the salt concentration and the application time were important factors determining the success of that method. Minimum use of culture supplies is also encouraged when the environment is radically controlled to support photoautotrophic system (i.e. by introducing forced ventilation, elevated carbon dioxide concentrations and removal of sugar from the media). Such systems not only produce superior plant quality in many cases, but also reduce waste of sugar and other supplies. Light must be augmented for these environmentally modified systems. However more efficient lighting systems are available to provide enhanced light intensity with reduce electricity consumption (KOZAI & SMITH, 1995). Strategies for reducing cost, control of environmental factors for promoting the autotrophic growth of the plantlets *in vitro* and for reducing the physiological disorders at the multiplication and preparation stage are discussed by KOZAI (1988).

1.4. Bioreactors

1.4.1. Bioreactor types and functions

A bioreactor is a self-contained sterile environment which capitalizes on liquid nutrient or liquid/air inflow and outflow systems. It is designed for intensive and frequently scaled-up culture and affords maximum opportunity for monitoring and control over micro-environmental conditions (e.g. agitation, aeration and temperature) (HARRELL, BIENIEK, HOOD, MUNILLA & CANTLIFFE, 1994). Bioreactor systems were traditionally used for bacterial fermentation or for large-scale production of secondary metabolites from plant cells (HEYERDAHL *et al.* 1995). Bioreactors are now being used for:

- somatic embryogenesis i.e. automated harvest of somatic embryos from a suspension culture in a bioreactor (STYER, 1985; PREIL, FLOREK, WIX, & BECK, 1988; HARRELL *et al.* 1994; MORRIS, SCRAGG, SMART, & STAFFORD, 1985; ETIENNE, 2000; INGRAM & MAVITUNA, 2000)
- obtaining secondary products (HOLDEN & YEOMAN, 1987; MORRIS *et al.* 1985; HEYERDAHL *et al.* 1995; FUKUI & TANAKA, 1995)
- cell cultures (MORRIS *et al.* 1985; FUKUI & TANAKA, 1995)
- micropropagation (BORROTO, 1997; TAKAYAMA & AKITA, 1998)
- meristem culture (ZIV, 1998; ZIV, RONEN & RAVIV, 1998).

The different applications of bioreactors in plant propagation have been documented (PREIL, 1991) and TAKAYAMA (2002) summarized the types of bioreactors used for mass propagation of different species and propagule types. There are various configurations of bioreactor systems, from single bubble column systems, through air driven draught tubes and loop rings to classical stirred reactors (FOWLER, 1988). Life Reactor ™ (OSMOTEK, 1998), shaker flasks, airlift, stirred tank, tower, packed column, flushed beds, hollow fiber, temporary immersion systems, cell-lift impeller, roller-bottle, stirred jars (MORRIS *et al.* 1985; FUKUI & TANAKA, 1995; HONG, LABUZA, & HARLANDER, 1989) and MISTIFIER™ bioreactor system using nutrient mists (WEATHERS, CHEETHAM & GILES, 1988) are some of the different types of bioreactors being used for plant culture. A number of automated systems based on the addition and removal of liquid media have been developed. The first system, described by TISSERAT &

VANDERCOOK (1985), consisted of a culture chamber for plant growth with inlets and outlets for nutrients. The principle has been used since in other automated systems (AITKEN-CHRISTIE & DAVIES, 1988), and SIMONTON, ROBACKER & KRUEGER (1991) attempted to make a fully programable micropropagation bioreactor. In bioreactors, large volumes of plants can be handled. Environmental factors such as the composition of the gas phase and pH can be controlled and factors affecting growth can be investigated in bioreactor systems (HOHE, WINKELMANN & SCHWENKEL, 1999; INGRAM & MAVITUNA, 2000). HVOSLEF-EIDE, OLSEN, LYNGVED & HEYERDAHL (2002) have developed a computer-controlled bioreactor for somatic embryo production. In this bioreactor temperature, oxygen, pH, stirrers (direction and speed) and light are controlled. Table 1.2 presents several bioreactor types used for the production of different species of plants. This table also describes the results that have been achieved using the different bioreactor types.

Table 1.2. Results of the use of different bioreactors for different plant species

Type of Bioreactor	Plant used	Results obtained			Reference
RITA®	*Elaeiss guineensis, Eucalyptus globulus* and *Solanum tuberosum*	Good multiplication rates			TEISSON, ALVARD, BERTHOULY, COTE, EXCALANT, ETIENNE & LARTAUD, 1996
RITA®	Rubber tree (*Hevea brasiliensis* (Müll.Arg)		Semi-solid	Bioreactor	ETIENNE *et al.* 1997
		No. Embryos per g fresh matter of callus	36	255	
		Shape of embryos (% of whole embryo)			
		Heart	14	4	
		Cotyledon	26	85	
		Abnormal	60	11	
		Increased root development and epicotyl emergence with the RITA®			
RITA®		Control (normal multiplication method of multiplication)		Multiplication rates in bioreactors	BORROTO & ETIENNE, 1998
	Pineapple	9		70	
	Sugarcane var 91301	3.6		36	
	var c105173	4.2		50	
	Syngonium	6.8		28	
RITA®	Potato var. Bintje	30 days from a single node gave			TEISSON & ALVARD, 1998
			Semi-solid	Bioreactor	
		No. Nodes	8.9	10.3	
		Height	8.4	13	
RITA®	Aspen *(Poplus tremuloides x P. Tremula)*	Per RITA® unit the number of shoots 208 and 334 after 6 weeks. Shoots were bigger and sturdier in the liquid system compared to the semi-solid system. Rooting of the shoots was successful out of the RITA® system. Aspen clones were more cost effective due to reduction in manpower and reduced media costs			KOKKO, HÄIKIÖ & KÄRENLAMPI, 2002
RITA®	*Psidium guajava* L. cv. Cuban red dwarf		Semi-solid	Bioreactor	KOSKY, PEROZO, VALERO & PEÑALVER, 2002
		Somatic embryo germination	9.8 %	91 %	
		Fresh weight	1.03 g	1.22 g	
RITA®	Apple	Used different plant growth regulators to find optimal multiplication. High BAP and Kinetin caused hyperhydricity			ZHU, LI & WELANDER, 2002
RITA®	*Narcissus Pseudonarcissus* cvs	Callus grew up to six times in volume in 14 weeks and organized structures developed			SAGE & SCHROEDER, 2002

Table 1.2. Results of the use of different bioreactors for different plant species

Type of Bioreactor	Plant used	Results obtained	Reference
Temporary immersion system (TIS)	Sugarcane	Increased shoot formation and shoot height.	LORENZO, GONZÁLEZ, ESCALONA, TEISSON, ESPINOSA & BORROTO, 1998
TIS	Pineapple	Used *in vitro* shoots as starting material. Comparison of solid, liquid and temporary immersion system was done. The immersions increased the multiplication rates.	ESCALONA *et al.* 1999
TIS	Internodes and micro tuber stage of potatoes	Found a 3 fold increase in shoot length and more internodes per plant and improved vigour. Growth rate of potato shoots was with immersions of 5 min every 3 h, 8 immersions per day. Fewer immersions per day resulted in reduced plant growth. Induced more tubers per plant than on solid medium but also increased the size and weight of the tubers. Tubers can be stored and directly transplanted without an acclimatization stage.	JIMÉNEZ, PÉREZ, DE FERIA, BARBÓN, CAPOTE, CHÁVEZ, QUIALA & PÉREZ, 1999
TIS	*Pyrus communis* var pyraster L. (wild pear)	Multiplication rate with TIS was 13 times whereas with the semi-solid was 4-5 times. Plants had excellent stem elongation and a higher rooting ability	DAMIANO, CABONI, FRATTARELLI, GIORGIONI, LIBERALI, LAURI & D'ANGELI, 2000
TIS	*Coffea arabica*	High quality somatic embryos – sowed directly in soil with a conversion rate of 78 %	ETIENNE, 2000
10 l vessels – TIS	Banana (*Musa* AAA cv. Grand Naine	Excessive growth of shoots which limited the number of shoots – increased costs as handling large shoots is not as easy	ALBANY, VILCHEZ, JIMÉNEZ, GARCÍA, DE FERIA, PÉREZ, SARRÍA, PÉREZ & CLAVELO, 2002
TIS 5l vessels	*Phalaenopsis*	Semi-solid Three fold shoot multiplication after 8 weeks Bioreactor Multiplication rate of 25x with eight immersions per day for a 12 week period	PREIL & HEMPFLING, 2002
Totally covered bubbling system	*Musa acuminata* group AAA.	5.2x multiplication rate in the bioreactor but hyperhydricity occurred and shoots were fragile	ALVARD, COTE & TEISSON, 1993
Agitated liquid culture	Tea	Increase in volume of shoots when liquid was continually agitated	SANDAL, BHATTACHARYA & AHUJA, 2001
10 l glass bioreactor	*Spathiphyllum*	30 000 plants obtained in two months and 3000 were transferable to the soil	TAKAYAMA & AKITA, 1994
1 l liquid medium flasks	Douglas-fir	Reduced labour costs. From 1l of liquid medium obtained over 10 000 somatic embryos	GUPTA, 2002

Table 1.2. Results of the use of different bioreactors for different plant species

Type of Bioreactor	Plant used	Results obtained		Reference
2 l bioreactor	Potato Poplar	An inoculum of 15 g bud clusters increased to 165 g in 30 days *In vitro* roots induced to form shoots increased in biomass from 2 g to 46 g (12 fold increase) after 30 days		ZIV, 2002
1 l twin flasks and 10 l scale up flasks	Banana Potato	Used paclobutrazol and ancymidol in temporary immersion Good compact bud clusters, very good rooting Temporary immersion more efficient than semi-solid – more tubers produced per plant and an increase in size and weight. Tubers produced in the bioreactors are able to be stored and planted straight to the field without an acclimatization stage		GONZÁLEZ, 2002
Bioreactor vessel with silicone tubes for bubbling and a vibration stirrer	*Euphorbia pulcherrima*	Cell doubling time 57 hrs in semi-solid and 40 hrs in the bioreactors		PREIL *et al.* 1988
Growtek bioreactor	Sandal wood (*Santalum album*) Chrysanthemum (*Dendranthema grandiflora*) Pineapple (*Ananus comosus*) Potato (*Solanum tuberosum*) Periwinkle (*Catharanthus roseus*)	Semi-solid 1.2 32 % 12 %	Bioreactor 21.4 times shoot production efficiencies 48 % minimization of root injury 18 % prevention of contamination loss	DEY, 2002
Disposable plastic bioreactors	Potato	Good multiplication		ZIV, 1998
Fed-batch bioreactor	*Lillium*	Periodic medium supplement into the cultures was an effective method for mass production (cut labour costs and was time saving)		SEON, KIM, SON & PAEK, 2000
	Musa acuminata (banana)			ALVARD *et al.* 1993
Gelled medium		Multiplication of 2.2 times		
Liquid medium with immersion		Proliferated a little or not at all		
Liquid medium with cellulose supports		Proliferated a little or not at all		
Liquid medium with partial immersion		Multiplication of 3.1		
Liquid medium aerated by bubbling		Multiplication of 3.1		
Liquid medium - TIS (20 min immersion every 6 hrs)		Multiplication rate greater than 5		

Table 1.2. Results of the use of different bioreactors for different plant species

Type of Bioreactor	Plant used	Results obtained				Reference
TIS, Roller-bottle Continuous immersion	Lily (*Lillium*)	The continually immersed state showed better growth than those periodically immersed or cultured in roller bottles.				TEISSON & SEON, 1999
	Tea Clone 'TRI-2025	Mean increase in mass	Mean total number of somatic embryos obtained			AKULA *et al.* 2000
			Globular	Maturing	No. of emblings	
Semi-solid		9.39	78.4	10	6.2	
Partial immersion using roller drum and liquid medium		5.65	16.4	6.2	5.8	
Continuous full immersion in shaker flask		1.75	15.2	5.0	0	
TIS (100ml liquid immersed for 1-2 min every 6 hr)		19.89	487.6	138	36	
	Phalaenopsis	Mean number of protocorm-like bodies				YOUNG, MURTHY & YOEUP, 2000
Air lift balloon bioreactor		10.8				
Air lift column bioreactor		6.1				
TIS		9.0				
TIS with charcoal filter		17				
Standard eramyler flask		9.2				
		A serious problem in liquid cultures was high frequency of hyperhydrated plants when organogenic propagules like bulblets, corms and micro-tubers and shoots were used as explants				
	Plum, Peach, Cherry, Apple	Multiplication rates				DAMIANO, LA STARZA, MONTICELLI, GENTILE, CABONI & FRATTARELLI, 2002
		Plum	Cherry	Peach	Apple	
Liquid		3 x	2 x	3 x	3 x	
Semi-solid		30 x	5 x	20 x	10 x	
30' immersion time (TIS)			6 x	8 x	2 x	
60' immersion time (TIS)		28 x	4 x	14 x	8x	

Table 1.2. Results of the use of different bioreactors for different plant species

Type of Bioreactor	Plant used	Results obtained					Reference
	Olive tree	No of new shoots/explants	% buds	Avg. Shoot height (cm)	Hyperhydricity	Callus	GRIGORIADOU, VASILAKAKIS & ELEFTERIOU, 2002
Semi-solid		1.78	89	1.2	---	-++	
Life reactor		0.35	18	0.4	-++	---	
Shaker		1.93	97	0.4	+++	---	
Filter bridges		0.43	22	0.32	---	+++	
TIS 30 days		1.93	97	0.93	--+	--+	
TIS 10 days + 20 days agar		0.64	32	1.01	---	-++	
TIS 20 days + 10 days agar		1.41	71	0.70	---	-++	
	Cymbidium	Multiplication of protocorms		Necrosis of protocorms		Plantlet hyperhydricity	PAMFIL, 2002
Agar solidified		3.6		2.3		3.8	
Liquid stationary		4.1		3.7		6.2	
Liquid agitated		4.6		3.5		8.5	
Bioreactor		5.1		5.3		11.8	
	Charybdis sp.	Total regenerates per gram	Shoots per gram	Shoots per container	Hyperhydricity (score 0-3)		WAWROSCH, KONGBANGKERD, KÖPF & KOPP, 2002
Agar media		229.0	158.3	158.3	0		
Liquid culture		59.5	54.8	130.4	3		
TIS (1 x 5 min/24h)		38.3	25.4	270.5	1		
TIS (2 x 5 min/24h)		48.0	36.6	437.0	3		
Automated plant culture system (glass bottle reservoirs, impeller pumps, plant culture chamber – computer controlled medium introduction aerated and continuously bathing the plants		Plant material obtained from semi-solid		Plant material obtained from bioreactor			TISSERAT & VANDERCOOK, 1985
	Potinera sp. Hybrid	17.1 cm^3		27 cm^3			
	Callistephus hortiensis (aster)	0.89 cm^3		1.8 cm^3			
	Pheonix dactyliferal L. cv. 'deglet Noor' (date palm)	4.29 cm^3		13.65 cm^3			
	Daucus carota L. Danvers	7.57 cm^3		14.67 cm^3			
	Mitragyna inermis O. Kuntze (cow tree)	0.69 cm^3		1.22 cm^3			

1.4.2. Environmental and chemical factors influencing choices of bioreactors

Particular species and different tissue types may be sensitive to a liquid medium environment in a detrimental way (AITKEN-CHRISTIE *et al.* 1995), whereas many other plant species exhibit improved performance when cultured on a liquid based medium as compared with a semi-solid medium (GAWEL & ROBACKER, 1990). Bioreactor culture is a highly effective means of mass propagating horticultural plants. The number of single nodes obtained by one-time cultures can be maximized since physical and chemical culture environments (air, light, gaseous supply, nutrient composition and number of plants in initial culture) can be controlled in optimal conditions (HAHN & PAEK, 2002). Sensitivity of plants cells to stress (mechanical, osmotic and nutritional), and changes of temperature, pH and O_2 tension, greatly limit the choice of immobilization methods, which can be used (MORRIS *et al.* 1985).

The decision to use one of the numerous different bioreactors that have been developed is specific for the requirements e.g. secondary products, multiplication of shoots, or somatic embryogenesis (AITKEN-CHRISTIE *et al.* 1995). To obtain secondary products the bubbled column system and stirred reactors were used (FOWLER, 1988). FUKUI & TANAKA (1995) employed an envelope shaped film culture vessel made of fluorocarbon polymer film, which was permeable to oxygen so agitation of the system was unnecessary. The size and shape of containers, methods of sealing and the type of lid, location of entrance to container, relationship of lid size to container bottom size, type of support system for shoots or plantlets, form of nutrients supplied, positions of entrances and withdrawal of nutrients, frequency of nutrient application, nutrient recycling, type of pump, type and size of nutrient reservoirs, and control for the system are all important in the choice of the bioreactor utilized (ALVARD *et al.*1993). The work undertaken by INGRAM & MAVITUNA (2000) reinforced the argument that bioreactors can be used successfully for large-scale somatic embryogenesis as long as the bioreactor configuration, design and operation conditions are carefully chosen to suit the physiological, metabolic and morphological characteristics of the culture. Common features to all containers are that they are clear, autoclavable and larger than conventional micropropagation containers (ALVARD *et al.* 1993). JIMÉNEZ *et al.* (1999) and BERTHOULY & ETIENNE (2002) stressed the importance of immersion frequency and duration for shoot multiplication and

development. These factors affect nutrient supply and composition of the internal atmosphere in the culture vessel.

The use of liquid medium can introduce the problem of asphyxia of explants as a result of immersion. The most commonly used preventative methods are based on the principle of partial immersion of explants to ensure aeration. Inert absorbant substances are used to maintain contact between the medium and the lower part of the explant or alternately a depth of medium is used to enable partial emergence of the explant tissue (ALVARD *et al.* 1993). HOHE *et al.* (1999) undertook a study to determine the effect of oxygen partial pressures in bioreactors on cell proliferation and differentiation in somatic embryos. They concluded that oxygen partial pressures could affect the proliferation of embryos of different cell lines. More importantly HOHE *et al.* (1999) found that attention must be paid to the pH of the cultures and the interaction of pH with other environmental factors. They found that the pH had a marked effect on the responses of the cell lines under different oxygen partial pressures. Differences in growth occurred with and without aeration. The shortage of oxygen led to small explants being produced in the liquid medium, thus suggesting that oxygen is a major limiting factor of growth. Lack of culture agitation led to asphyxiation of the explant. Control of the duration of immersion of the explants requires the most attention in the design of culture systems in liquid medium with temporary immersion. The temporary immersion culture system described by ALVARD *et al.* (1993) combines the ability to aerate plant tissue and provide contact between the whole of the plant and liquid medium. These two features are not combined in classic liquid culture procedures. Bioreactor systems all improve the nutrition and gas exchanges and thus most of these systems improve the quality of the propagules (size, etc.) (Table 1.2).

MARTRE, DOMINIQUE, JUST & TEISSON (2001) stated that the major problem with the use of liquid medium is hyperydricity, a severe physiological disorder involving apoplastic water accumulation due to extended contact between the explants. Hyperhydricity frequently occurs with tissues grown in or on liquid medium, as a result of contact with the liquid and other micro-environmental parameters present at the time. Submersion of tissues readily induces hyperhydricity in some cases. MAJADA (1998) determined that high ventilation rates not only reduced hyperhydricity in the logarithmic growth phase, but also induced a reversion of hyperhydric shoots, favouring formation of normal new shoots.

The absence of a gelling agent may increase availability of water and dissolved substances to the explant (DEBERGH, 1983). Bioreactors for larger explants (including elongated shoots, plantlets or germinated somatic embryos) typically have been of a double layer type whereby the liquid nutrient solution bathes the root or base of the shoot/s and the leaves develop further in a more natural gaseous atmosphere. HAHN & PAEK (2002) discovered with the use of *Chrysanthemum* shoot cultures that the type of medium supply (ebb and flood, immersion, rafts) was important in shoot multiplication in bioreactors. The temporary immersion type of bioreactor was better for normal leaf and stem development, avoided hyperhydricity and reduced asphyxiation of tissues, and was more suitable for acclimatization and development towards photoautotrophy (AITKEN-CHRISTIE *et al.* 1995; CIRAD 1999).

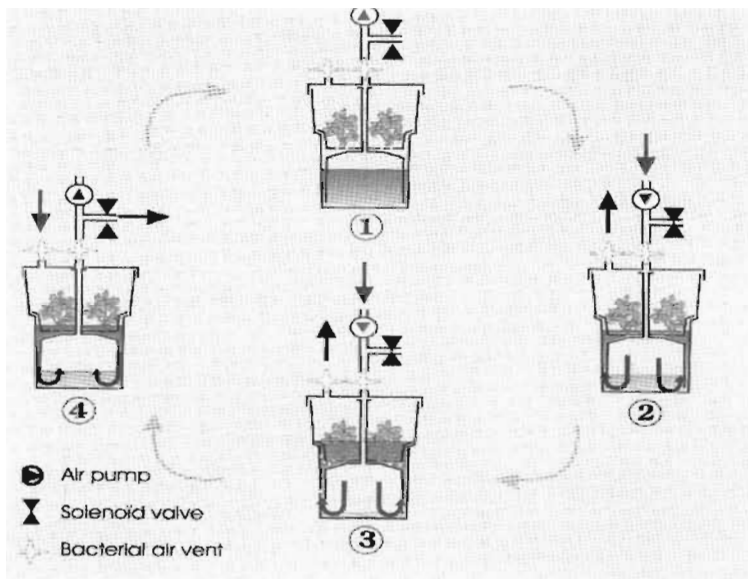
KOZAI & SMITH (1995) found that *in vitro* plants which became photoautotrophic, had increased growth, decreased physiological and morphological disorders, uniform growth and development, and rooting and acclimatization were more readily achieved. Furthermore, the use of a bioreactor allowed control over the air movement, which facilitated in photoautotrophy of the plants that, in turn allowed a reduction of sugars in the media (which can reduce the loss of cultures due to contamination).

1.4.3. Temporary immersion bioreactor system (RITA®)

The advantages of liquid media for enhancing shoot propagation (HARRIS & MASON, 1983), growth (SKIDMORE, SIMONS & BEDI, 1988) or somatic embryogenesis (JONES & PETOLINO, 1988; GAWEL & ROBACKER, 1990) has been reported for several species. The precise mechanisms involved in this improved performance are not known (ALVARD *et al.* 1993). To avoid the problems associated with both semi-solid and liquid media, different procedures have been developed. Among these, the temporary immersion of explants in a liquid medium has been achieved by using different bioreactors. The use of the temporary immersion bioreactor system (RITA®) has been reported to contribute to increased yields of many species of plants, when compared with conventional (semi-solid) micropropagation systems (Table 1.2).

The RITA® system consists of two compartments (Figure 1.1). The upper chamber contains a polyurethane foam disc on a nylon disc onto which the explants are placed. The lower

compartment contains the medium. The two compartments are linked in such a way that when an overpressure of 30 Kpa is applied in the lower compartment the medium is pushed into the upper compartment. The overpressure escapes through filtered outlets (0.22 μm hydrophobic bacterial air vents) in the lid of the apparatus. During the emmersed stage a flow of air bubbles through the medium lightly agitates the explants and renews the atmosphere inside the culture vessel. Due to the hydrophobic nature of the bacterial air vents and the communication between the two compartments of the apparatus, the explants are kept in a water-saturated atmosphere even when emmersed. A pump generates the overpressure and an unequal cyclic timer controls the frequency and duration of the immersed stage.



1. Plants are placed on a polyurethane foam disc in the upper compartment. Plants are at rest or standby (i.e. the nutrient is in the lower compartment). This is the longest stage.
2. Overpressure of sterile air is applied in the lower container pushing the nutrients up into the upper compartment, immersing the explants.
3. The immersed stage is a sterile airflow continuously agitating and oxygenating the medium and renewing the air in the vessel. The flooding stage has the shortest duration. Optimum duration of flooding and rest has to be determined empirically.
4. When the airflow ceases, the pressure in the two parts of the container adjusts and the liquid medium returns to the bottom of the vessel by gravity. The plants remain covered by a film of medium by capillary attraction. (CIRAD, 2002).

Figure 1.1. Operating cycle of the RITA® system

RITA® is designed to immerse the plant with media temporarily for a few minutes. The duration of immersion and time between immersions is controlled and can be regulated (BAURENS, 1998; VITROPIC CIRAD, 2000). MARTRE *et al.* (2001) looked at the relative growth rate,

water content, the CO_2 production and the total energy change at different times of immersion and comparisons were made between the semi-solid system, an agitated liquid medium system and a temporary immersion system (RITA®). The researchers found that the relative growth rate of callus of *Hevea brasiliensis* decreased with increased immersion times. During immersion the rate of respiration was the same for the different treatments but at the rest time the rate of respiration increased. Unlike growth conditions in semi-solid and agitated liquid media, the temporary immersion (RITA® system) induced severe oxidative stress in callus of *Hevea brasiliensis* at the immersed stage. An immersion as short as one minute was enough to cause a high lipid peroxidation, which disappeared in less than one hour after the end of the emmersed stage. The link between good growth conditions during the emmersed stage and short-term stress conditions during the immersed stage may explain the previous success achieved with somatic embryogenesis in the temporary immersion system. It is necessary to define precisely the duration and frequency of immersion for the particular species. MARTRE *et al.* (2001) showed that temporary immersion is associated with stressful conditions, which could explain why conditions under which the explants are cultivated in the RITA® system are critical. AKULA *et al.* (2000) discovered that the immersion time interval impacted significantly on the multiplication rates of somatic embryos in the temporary immersion system and an increase or a decrease in the immersion frequency played a decisive role in influencing the multiplication rates. One minute every six hours was most beneficial for somatic embryos. In liquid cultures, unlike semi-solid agar cultures, the entire surface area of the explant comes into uniform direct contact with the medium allowing more efficient uptake of nutrients. Toxic metabolites, which may accumulate in the vicinity of the tissue, are more effectively dispersed by liquid immersion.

ESCALONA *et al.* (1999) found that in some species the multiplication rates could increase by 300-400 % in the RITA® system. However, many of the shoots produced were not adequate in size for *ex vitro* rooting so a further elongation stage was necessary. A partial explanation for the good plant growth in the RITA® system could be the effect on the physiology of the explants due to the environmental conditions. Nutrient supply and composition of the internal atmosphere in the culture vessel could contribute to the health of the plants. With the RITA® system there was improved nutrition as the medium was in direct contact with the plants during immersion and a capillary film covered the plants throughout the remaining period. There was marked reduction in asphyxiation and hyperhydricity compared with permanent immersion, there was complete

renewal of atmosphere at each immersion, and tissue division occurred during agitation due to bubbling. The system allowed control of the morphological process through modification of frequency and duration of immersion, and air vents guaranteed protection of each apparatus. Individual handling was possible, and there was minimal risk of spreading of contamination (BORROTO, 1997; CIRAD, 2002). AKULA *et al.* (2000) found that callus formation, hyperhydricity and other developmental abnormalities were not observed at any stage in the process using the temporary immersion system (RITA®) for somatic embryogenesis and plant recovery in tea clones. Plants produced using this method were successfully acclimatized to greenhouse conditions.

In addition to improved multiplication rates and quality of micropropagules, the RITA® system greatly reduced material and labour costs (ETIENNE *et al.* 1997; BORROTO & ETIENNE, 1998). ALVARD *et al.* (1993); BORROTO (1997), BORROTO & ETIENNE (1998), CIRAD (1999), and VITROPIC CIRAD (2002) reported that the temporary immersion bioreactor system (RITA®) has many advantages:

- great reduction in cost of media used
- less space utilized to produce more plants
- no cost of gelling agent
- less ecological problem of discarding old media
- labour saving as there is a reduction in handling of plants and media; no cleaning of agar off the plants
- shorter sub culturing time for explants (since the explants do not require positioning in the medium but are simply placed in contact with it)
- complete renewal of the atmosphere at each immersion
- the composition of the medium can be changed by simple transfer
- survival rate at hardening-off is reported to be improved
- suitability for industrial and research purposes

In the production of somatic embryos of coffee, ETIENNE (2000) found that by using the temporary immersion bioreactor system there was an elimination of the *in vitro* plantlet stage which is most labour intensive as direct sowing of coffee embryos into the soil was possible thus reducing the costs. Using this system, ETIENNE (2000) stated that three months *in vitro* culture

time was saved, together with a 6.3 % reduction in handling time compared with that of plantlets produced using a semi-solid method. The use of the temporary immersion system for the production of somatic embryos in woody species, together with the sowing of these uncoated somatic embryos, represents an alternative to the production of artificial seeds and may provide an economically viable solution for many species (ETIENNE, 2000). TEISSON (1998) found that the quality of plantlets produced in the temporary immersion bioreactor allowed direct transfer to the nursery for growing and hardening. The number of embryos of coffee produced in the semi-solid system was six to 200 while 9000 were produced with the RITA® system. Using the semi-solid system it took 80 hours for handling 10 000 plants whereas using the RITA® system 10 000 plants were handled in 0.2 hours (CIRAD, 2002). It was proposed that one of the reasons for the efficiency of the temporary immersion culture system was possibly the ability of the system to aerate plant tissue and provide contact between entire explant and liquid medium. In addition it was found that the volume of the medium placed in the system had an influence on the multiplication rate, with higher than 200 ml causing the multiplication rate to decline. With pineapple culture it was found that multiplication was greatest at the seventh week of growth when the pH was stable at 3.5. One of the advantages of temporary immersion culture on *in vitro* nutrition may be that temporary immersion limits the movement of ions out of the plants, which is associated with the pH change (CIRAD, 2002). There is an initial net decrease in the mineral content of plants following the transfer to fresh medium during each subculture in conventional micropropagation (ESCALONA *et al.* 1999). The temporary immersion system combines the advantages of semi-solid and constant immersion while avoiding the problems such as hyperhydricity and asphyxia (AKULA *et al.* 2000). Plant material propagated by temporary immersion performs better during the acclimatization phase than material obtained on semi-solid or liquid media. Successful regeneration of plants after direct sowing in soil of *Solanum tuberosum* micro tubers and *Coffea arabica* somatic embryos produced in temporary immersion bioreactors, has been demonstrated (BERTHOULY & ETIENNE, 2002).

In conclusion, the statements made by SKIDMORE *et al.* (1988); BERTHOULY & ETIENNE, (2002) and many other researchers in relation to the advantages of the temporary immersion system that there was:

- a reduction of labour costs
- simplified handling of plants and medium

- improved nutrition
- marked reduction in asphyxiation and hyperhydricity
- complete renewal of atmosphere at each immersion
- tissue division occurred during agitation due to bubbling
- control of morphological process though modification of frequency and duration of immersion
- production of healthy acclimatized plants

are well supported by their results. The literature shows that the bioreactor systems can enhance all the characteristics needed for good growth and development of plants. It is these factors that led to the study of the temporary immersion bioreactor to determine its value for the production of *Eucalyptus* clones *in vitro*.

Chapter 2. Establishment of Shoots and Control of Contamination in the Temporary Immersion Bioreactor (RITA®)

2.1. Introduction

Woody species have a high microbial infection rate and it has been found that fungal and bacterial infections can vary according to the season and other environmental conditions, thus it is difficult to obtain clean plant material for establishment *in vitro*. DAS & MITRA (1990) found the best season to be July to September (Northern hemisphere) for the harvest of the explant source for rapid and increased multiplication of axillary buds of *Eucalyptus tereticornis* Smith. However, this would be different in South African conditions. Ideally *in vitro* contaminants should be identified so the correct preventative measures can be used (CASSELLS, 1991; REED & TANPRASERT, 1995). CASSELLS (1997) and HOLDGATE & ZANDVOORT (1997) summarized various screening methods and disease indexing which could be used to eliminate contamination at different stages of micropropagation. However, this is time consuming and a labour intensive process and is often not feasible in a production laboratory. With *Eucalyptus* production at Mondi Forests, there are many different clones being produced at any given time and screening of all of them would slow down the production process, so it is important to eliminate microbial contamination prior to or at the culture initiation stage for the cost efficacy of the entire program.

The objectives behind the elimination of microbial contamination and the maintenance of an aseptic environment are to obtain pathogen free plants and to eliminate or minimize the death or degradation of plants caused by contamination during *in vitro* culture. The first step of eliminating microbial contamination is the choice and pre-treatment of the mother plant. Thereafter the selection of explant type and sterilization is important (DEBERGH & VANDERSCHAEGE, 1990). Once contaminant free explants have been obtained it is necessary to maintain the cultures in this state. However, many plants have endogenous micro-organisms and these have adverse effects on cultures as the micro-organisms use up the nutrients and multiply faster than the plants which causes death of the plants. These endogenous contaminants can grow at any stage throughout the culture especially if the plants are under stress. Contamination may also be introduced into cultures during transfers. Thus it is imperative to

find methods to maintain pathogen free cultures, prevent introduction of new contaminants and to cure both endogenous fungal and bacterial contamination (LEIFERT, 2000). It should be noted that, the micro-organisms which cause microbial contamination and the death of plants *in vitro* are often not the pathogens which cause diseases of plants in the field or in the greenhouse (KOZAI & SMITH, 1995).

This chapter discusses aspects of establishment of different types of shoots into a temporary immersion bioreactor (RITA®) system, together with the necessity of maintaining microbial free cultures, which are essential when working with large numbers of shoots in the bioreactor. It is important to find the optimal method of placement of shoots into the RITA® vessels, and the correct fungicides and antibiotics to use to eliminate or reduce contamination *in vitro* in the RITA® system.

2.2. Materials and Methods

2.2.1. Establishment of axillary buds into RITA® vessels

The following establishment treatments were tested (six clones per treatment were used due to the clonal specificity of nutrients and response *in vitro*, three vessels of 20 shoots per vessel per clone). At all stages the plants were grown in growth rooms at 22-25 °C, at 16 hours light/ eight hours dark photoperiod. Cool white flourescent lights illuminated growth rooms (1 360 lux). After each treatment percentage contamination was recorded.

a. Nodal explants – introduction into RITA® vessels

Potted parent plants were sprayed with Sporgon® and Bravo® prior to harvesting (parent plant pre-treatment). Single nodal explants with reduced leaf area (¼ area) were prepared, submerged and aerated for three hours in Benlate® (1 $g.l^{-1}$) and boric acid (1 $g.l^{-1}$). The explants were surface-sterilized with calcium hypochlorite (2 $g.l^{-1}$) for five minutes and then placed in a solution of mercuric chloride (0.1 $g.l^{-1}$) and two drops of Tween® 20 for two minutes. After which they were washed three times with sterilized water and rinsed with Bravo® (1 $ml.l^{-1}$). This was the standard sterilization protocol developed for *Eucalyptus* initiation at Mondi Forests. The explants were placed into RITA® vessels containing multiplication media (M1, Appendix 2).

The media for this study and all the further studies were made up to a pH of 5.8 and autoclaved at 121 °C at a pressure of 1 Kpa.

b. Secondary leaders from rooted cuttings in the greenhouse – introduction into RITA® vessels

Secondary leaders from rooted cuttings in inserts were excised. These were sterilized with 0.5, 1 and 2 $g.l^{-1}$ calcium hypochlorite or 1.2 %, 1.75 %, and 3.5 % (v/v) sodium hypochlorite for 5, 10 and 15 minutes, and rinsed three times with sterile distilled water. The secondary leaders were then placed into RITA® vessels containing multiplication media (M1, Appendix 2).

c. Axillary bud placement into RITA® vessels via a semi-solid phase

Nodal explants taken from parent plants (treated as in treatment a) were sterilized (as in treatment a). The sterilized shoots were then placed onto semi-solid initiation media in jars (Appendix 2), to enhance axillary shoot growth and extension. All fungal contamination was eliminated at this stage. When the axillary buds from the nodal sections were about 1-2 cm long they were excised and placed into semi-solid multiplication media (M1, Appendix 2) in jars. After 14 days on semi-solid M1 media, contaminant free shoots were visually selected and placed into RITA® vessels containing liquid M1 (Appendix 2).

d. *In vitro* shoots from the semi-solid media introduced into the RITA® vessels

Established (five months old) multiplying shoots *in vitro* which were visually contaminant free were selected, removed from the semi-solid M1 and placed into RITA® vessels containing liquid M1 medium (Appendix 2).

e. Axillary bud placement into RITA® vessels via a semi-solid phase and a seven day treatment of Rifampicin

Nodal explants sterilized as in treatment a, were placed onto initiation media (Appendix 2) to enhance axillary shoot growth and extension. All fungal contamination was eliminated at this stage. When the axillary buds from the nodal sections were about 1-2 cm long they were excised and placed into semi-solid multiplication media (M1, Appendix 2) in jars. After 14 days on the semi-solid multiplication media, contaminant free shoots were visually selected and placed in 0, 0.01, 0.05, 0.1, 0.2 $g.l^{-1}$ Rifampicin on a shaker for seven days at 70rpm. Contaminant free shoots were selected and placed into RITA® vessels containing liquid M1 media (Appendix 2).

f. *In vitro* shoots from a semi-solid media with a Rifampicin treatment prior to introduction into the RITA® vessels

Visually contaminant free multiplying *in vitro* shoots (five months on multiplication media) were placed in liquid MS (Appendix 2) containing different concentrations (0, 0.01, 0.05, 0.1, 0.2g.l^{-1}) Rifampicin (shaking at 70rpm for seven days). Contaminant free shoots were selected visually five days after the Rifampicin treatment and placed into the RITA® vessels in multiplication media (M1).

2.2.2. Antibiotics and fungicides used as preventatives and curatives in the RITA® vessels after establishment of shoots

High losses occurred due to contamination throughout the multiplication stages and it was speculated that antibiotics and fungicides could be used to prevent or eliminate the occurrence of contamination in the cultures. The clones which were selected for the treatments were established and growing well in the RITA® vessels, some however had contamination and thus they were used for the different treatments. Fungicides or antibiotics at various concentrations (Table 2.1) were filter sterilized into the media in the RITA® vessels.

Table 2.1. Concentrations of fungicides and antibiotics used on different clones as curatives and preventatives for elimination of contamination

Fungicides	**Clones**	**Concentrations**	**Treatment**
Benlate®	GN107, GN108, NH58	0.1 g.l^{-1}, 0.5 g.l^{-1}, 1 g.l^{-1}	Curative/ Preventative
Bravo®	TAG31, GN107, GN108, GN121	0.5 ml.l^{-1}, 0.8 ml.l^{-1}, 1 ml.l^{-1}	Curative/ Preventative
Sporgon®	TAG31, GN107, GN108	0.1 g.l^{-1}, 1 g.l^{-1}	Curative/ Preventative
Sporekill®	TAG31, GN107, GN108, NH58	1 ml.l^{-1}, 2 ml.l^{-1}, 5 ml.l^{-1}	Preventative
Plant Preservative Mixture® (PPM)	GN108	1 ml l^{-1}, 2 ml l^{-1}, 5 ml l^{-1}	Curative/ Preventative
Puragene®	TAG31, GN108, NH58	0.05 ml.l^{-1}, 0.1 ml.l^{-1}	Curative/ Preventative
Antibiotics			
Rifampicin with sponges in the vessels.	TAG76, GN107, GN108, GN121, NH58,	0.01 g.l^{-1}, 0.02 g.l^{-1}, 0.1 g.l^{-1}	Curative/ Preventative
Rifampicin without the sponges	GU175, GU180, GN108	0.1 g.l^{-1}	Curative/ Preventative
Antibiotic cocktail	TAG31, GN107, GN108, NH58	Claforan 0.2 ml.l^{-1} Garamycin 0.2 ml.l^{-1} Novostrep 0.3 ml.l^{-1} Zinaceff 0.5 ml.l^{-1} Novocillin 0.34 ml.l^{-1}	Curative/ Preventative

Shoots were grown in the media containing the antibiotics and fungicides and contamination was recorded (preventative). Shoots from contaminated vessels were taken from the infected vessels and placed in new vessels with the different concentrations of different fungicides or antibiotics filter sterilized into the media in the RITA® vessels to determine whether contamination could be eliminated from these shoots (curative) (Table 2.1). The effect of antibiotics and fungicides on the plants and the contamination was recorded.

2.3. Results and Discussion

2.3.1. Initiation of shoots into RITA® vessels

Establishment of nodal cuttings of six different clones directly into the RITA® vessels was the first attempt at obtaining contaminant free cultures (treatment a, section 2.2.1). Although this material came from pre-treated parent plants, this method of initiation into the RITA® system was unsuccessful. There was 100 % contamination in all the vessels for the six different clones used (Table 2.2).

Table 2.2. Contamination occurring when nodal explants were sterilized and initiated directly into the RITA® vessels (treatment a, section 2.2.1) from shoots of six clones

Treatment a	GN107	GN108	NH058	TAG31	GU175	GU180	Average % Contamination for all clones
% Contamination	100	100	100	100	100	100	100

The use of nodal explants resulted in total contamination, as 20 nodes were placed per vessel and a single contaminated node could result in the whole vessel becoming contaminated. The method of sterilization used was the standard practice for initiation of nodal explants into the semi-solid media. Contamination percentages usually ranged from 20 % to 80 % dependant on the clone and if material was taken from pre-treated parent plants. Elimination of fungal and bacterial contamination is more difficult when the starting material for culture is taken from field grown plants (WARRAG, LESNEY & ROCKWOOD, 1990), or if the plant carries exogenous contaminants, which are not eradicated by conventional surface sterilization (WATT *et al.* 1996). It was not feasible to use nodal explants, as some of the plant material was older and therefore likely to have higher microbial counts than more juvenile material from mature trees. IKEMORI

(1987) found the average contamination rate to be 60 % if nodal sections of 58 *E. grandis* mother trees were used. This author found 37 % contamination rate if apical buds were used but necrosis of the buds occurred. The use of different explant material was needed to initiate shoots into the RITA® vessels.

Secondary leaders were taken as explants from rooted cuttings in the greenhouse (treatment b). They were sterilized using different concentrations of calcium hypochlorite and sodium hypochlorite and different periods of time (treatment b, section 2.2.1). The use of the secondary leaders as explants for establishment was however unsuccessful (Table 2.3).

Table 2.3. Contamination occurring using treatment b (section 2.2.1) to establish secondary leader shoots of six clones in RITA® vessels (three vessels per treatment of each clone were used)

Treatment b	**Calcium**	**Time in minutes**			**Sodium**	**Time in minutes**		
	hypochlorite	**5**	**10**	**15**	**hypochlorie**	**5**	**10**	**15**
Clone	**($g.l^{-1}$)**	**% Contamination**			**(%)**	**% Contamination**		
GN107	0.5	100	100	100	1.2	100	100	100
	1	100	100	100	1.75	100	100	dead
	2	100	100	dead	3.5	100	dead	dead
GN108	0.5	100	100	100	1.2	100	100	100
	1	100	100	100	1.75	100	100	dead
	2	100	67	67	3.5	100	dead	dead
NH58	0.5	100	100	100	1.2	100	100	dead
	1	100	67	67	1.75	100	dead	dead
	2	100	dead	dead	3.5	100	dead	dead
TAG31	0.5	100	100	100	1.2	100	100	100
	1	100	67	67	1.75	100	100	dead
	2	67	67	67	3.5	100	dead	dead
GU175	0.5	100	100	100	1.2	100	100	dead
	1	100	100	67	1.75	100	100	dead
	2	100	67	dead	3.5	100	dead	dead
GU180	0.5	100	100	100	1.2	100	100	100
	1	100	100	100	1.75	100	100	dead
	2	100	67	dead	3.5	100	dead	dead

Contamination (100 %) occurred in all the clones at 0.5 $g.l^{-1}$ of calcium hypochlorite, suggesting that this concentration was too low. However when 2 $g.l^{-1}$ for 10 and 15 minutes was used, GN108 and TAG31 had 67 % contamination (Table 2.3). At 1 gl^{-1} calcium hypochlorite TAG31 gave 67 % contamination indicating that a lower concentration for less time could be used on this clone. At 2 $g.l^{-1}$ for longer time periods death occurred with some clones (GU180, NH58 and GN107). This was due to the fact that the secondary material is young and cannot withstand a harsh sterilization regime. A clonal difference could be seen in the response to the sterilization

treatments with some clones being able to withstand higher concentrations of sterilants for a longer period of time than others.

Sodium hypochlorite at higher (3.5 %) concentrations for longer periods of time (10 and 15 min) caused death of the explant. However, the time period of five minutes exposure to the hypochlorite solutions at different concentrations was not long enough to eliminate surface microbes. When using soft young material it is difficult to obtain sterilization regimes that are rigorous enough to destroy the surface microbes without becoming toxic to the young shoots. The goal in surface sterilization is to remove all of the micro-organisms with a minimum of damage to the plant system to be cultured (CRESSWELL & DE FOSSARD, 1974; DODDS & ROBERTS, 1985). IKEMORI (1987) using *Eucalyptus grandis* epicormic shoots also found that contaminant free explants were difficult to obtain without killing the plant tissue when too high concentration of disinfectant was used. Further, some of the *Eucalyptus* clones are more sensitive to sodium hypochlorite than calcium hypochlorite. With *Eucaluptus grandis* IKEMORI (1987) found that the ability to survive rigorous sterilizations differed in explants from different trees.

In this study, placement of shoots directly into the RITA® vessels after sterilization resulted in a significant risk that a single contaminated shoot could infect the entire vessel. It became obvious that this method of initiation of shoots directly into the vessels would not be appropriate and a different approach had to be taken.

The semi-solid system was used for the introduction of nodal sections, prior to induction of shoots into the RITA® vessels (treatment c, section 2.2.1, pg 54). Nodes were sterilized and placed onto semi-solid initiation media. The axillary buds were excised from the nodes and put into semi-solid multiplication media for 14 days. A visual selection of these shoots was then done prior to transfer into the RITA® vessels (treatment c). This use of the semi-solid media facilitated the removal of fungal contamination (Table 2.4), which was the main cause of contamination in the previous initiation treatments (a and b). After placement of the visually contaminant-free shoots into the vessels, an average of 56 % bacterial contamination occurred across the clones (Table 2.4). GN107 and GN108 had 33 % contamination while the other clones had a higher contamination of 67 %. When treatment d (section 2.2.1, pg 54) was tested, where visually contaminant free, multiplying plantlets from *in vitro* culture (for five months)

were placed directly into the RITA® vessels, an average of 33 % bacterial contamination was obtained with the different clones used (Table 2.4). GN108 and NH58 had no contamination with GN107 having 33 %.

In conclusion, this approach proved successful for some clones but not others; it is not therefore, a totally reliable method as it does not circumvent the problem of endogenous contamination.

Table 2.4. Contamination occurring using treatments c and d (section 2.2.1) to establish shoots of six clones in RITA® vessels (three vessels per clone per treatment were used)

Clone	**% Contamination**	
	Treatment c	**Treatment d**
GN107	33	33
GN108	33	0
NH58	67	0
TAG31	67	67
GU175	67	33
GU180	67	67
Average % contamination for all clones	56	33

The bacterial contamination needed to be eliminated, as it was in competition with the multiplying shoots for nutrients and eventually the contamination utilized all the available nutrients and caused the death of the plantlets. Latent contamination reduces the reliability of plant tissue culture systems since small changes in the environmental conditions may allow rapid proliferation of the contaminants. These changes may be in temperature, pH, media or increased exudates which plants produce when grown at higher densities. This could result in reduced plant growth or rooting or even kill the plants (LEIFERT, 2000).

The use of an antibiotic in the media was undertaken as part of a pre-treatment to overcome the bacterial problem, which occurred in treatment c and d, was undertaken. According to REED & TANPRASERT (1995) plant tissue culture media reduce the effectiveness of antibiotics slightly and it is important to determine the minimum concentration of antibiotics required for maximum effectiveness, without becoming phytotoxic to the plants. Different antibiotics tested by other researchers showed toxicity and ineffectiveness (Table 1.1). PHILLIPS *et al.* (1981) and CORNU & MICHEL (1987) found Rifampicin to be an effective antibiotic with no phytotoxic effects to the plants. Thus it was used at various concentrations to determine if it would be effective against the bacterial contamination occurring in the RITA® vessels with the different

Eucalyptus clones. Various concentrations of Rifampicin were added to the liquid MS medium. Shoots were placed in jars in this medium for seven days on an orbital shaker (70 rpm). Cloudiness of the medium after the seven days was taken as a sign of the presence of bacterial contamination, and these shoots were then discarded (Table 2.5). Only the clean shoots were placed into the RITA® vessels.

Table 2.5. Contamination obtained prior to placement in the RITA® vessels using treatments e and f (section 2.2.1) for shoots of six clones

		% contamination					
Treatment	**Rifampicin ($g.l^{-1}$)**	**GN107**	**GN108**	**NH058**	**TAG31**	**GU175**	**GU180**
e	0	100	100	100	100	100	100
	0.01	100	66	66	100	66	100
	0.05	0	66	0	66	66	0
	0.1	0	0	0	0	0	0
	0.2	dead	0	dead	0	0	0
f	0	100	100	100	100	100	100
	0.01	100	0	66	0	0	100
	0.05	0	0	0	0	0	0
	0.1	0	0	0	0	0	0
	0.2	dead	0	dead	0	0	0

The controls (no Rifampicin) for both treatment e and f exhibited 100 % bacterial contamination. The use of 0.2 $g.l^{-1}$ of Rifampicin caused some necrosis of the shoots and death of GN107 and NH58 shoots. At 0.05 and 0.01 $g.l^{-1}$ Rifampicin contamination occurred with some of the clones. On the other hand 0.1 $g.l^{-1}$ Rifampicin resulted in contaminant free explants and had little effect on the shoots. All shoots with no visible signs of contamination were then placed into the RITA® vessels, after which no bacterial contamination occurred. Obtaining contaminant free shoots in RITA® by using the semi-solid medium and Rifampicin pre-treatment with visual selection of contaminant free plants is thus appropriate for the six *Eucalyptus* clones tested (Table 2.5). Rifampicin is a potent inhibitor of DNA-dependent RNA polymerase of bacterial and chloroplast origin (SIGMA CATALOGUE, 1994). The phytotoxic effects on the plants were visible on some of the clones at the higher concentrations where chlorosis and eventually death occurred.

BERTHOULY & ETIENNE (2002) stated that they always initiated plants into a semi-solid system prior to placement into RITA® vessels as they found losses were too high if plants were initiated into the vessels directly (without prior selection) and it was too costly to do pre-screening of the explants. ESCALONA *et al.* (1999) and PREIL & HEMPFLING (2002) used

established shoots from an agar base as inoculum for the bioreactors, as indicated by most other researchers. Similarly, with *Eucalyptus* clones it was important that elimination of contamination was undertaken in the semi-solid phase after which the shoots were then used for the liquid systems. Unless disease indexing of the parent plant or screening takes place, as described by CASSELLS (1997) and HOLDGATE & ZANDVOORT (1997), it is not possible to place shoots directly into the RITA® vessels without obtaining high losses. For the initiation of *Juglans, Prunus* and *Malus* species in a commercial laboratory, VISS, BROOKS & DRIVER (1991) used a 523 media and streaked the explants across the media (incubated for two days) and then used this as detection for the contaminant free explants. Such technique could be utilized for the initiation of *Eucalyptus* but would be time consuming, as each clone would have to be tested.

Explant contamination can represent the highest cost element of any micro-propagation operation when expressed in terms of labour, chemicals, energy and space (HOLDGATE & ZANDVOORT, 1997). Initiation undertaken directly into the vessels would be the most cost effective approach. Pretreatment via a gel system is time consuming and costly in that the use of gel causes increases in overall media costs. Continued use of antibiotics gives a higher risk of resistance by the micro-organisms and phytotoxicity to the plants and could become a serious health problem to people utilizing the antibiotics. It is therefore important to find methods that will obviate all the problems. Although the two step method of placement into semi-solid media and then an antibiotic treatment gave the best results so far, further studies need to be undertaken to find an inexpensive and efficient screening method for the different *Eucalyptus* clones.

2.3.2. Preventatives and curatives of fungal and bacterial contamination using fungicides and antibiotics

After culture establishment, as discussed above, the cultures were subcultured and maintained. However, a significant loss in numbers occurred after the third cycle of shoot multiplication in the vessels due to both fungal and bacterial contamination (Figure 2.1 and Figure 2.2). The fungal contamination that occurred could have been due to reduced efficiency of the 0.22μ filters, or the support sponges not being cleaned sufficiently, poor technique or latent endogenous contamination. At this point the potential for losses due to microbial contamination was determined to be far greater in the RITA® system (50 up to 300 shoots lost per contaminated

vessel) compared with the conventional semi-solid system (seven to 21 shoots lost per contaminated vessel). Losses with the RITA® therefore were substantial and finding curative or preventative methods of elimination was deemed crucial to the success of the method and its applicability to the industry. Consequently, fungicides and antibiotics were tested on different clones to determine their effectiveness as preventatives or curatives.

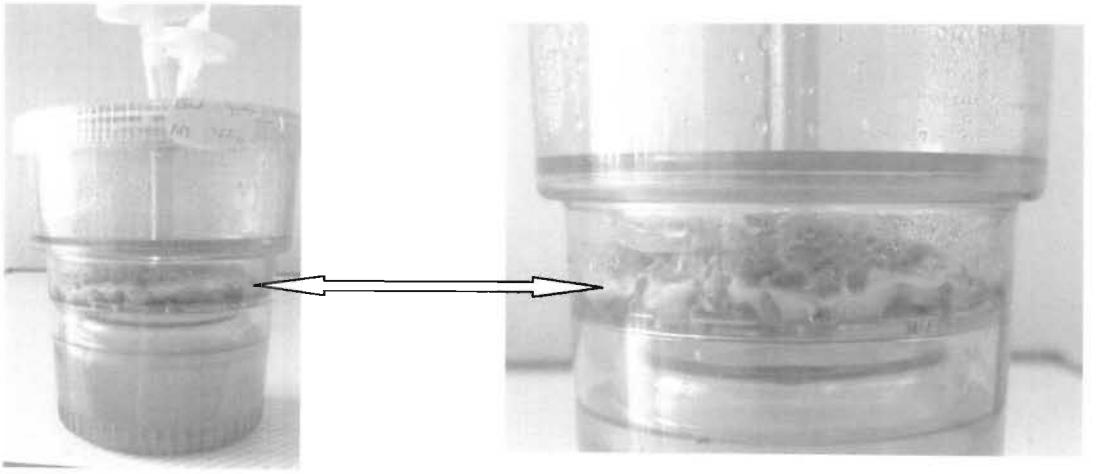


Figure 2.1. Fungal contamination in the vessels (arrow shows fungal contamination)

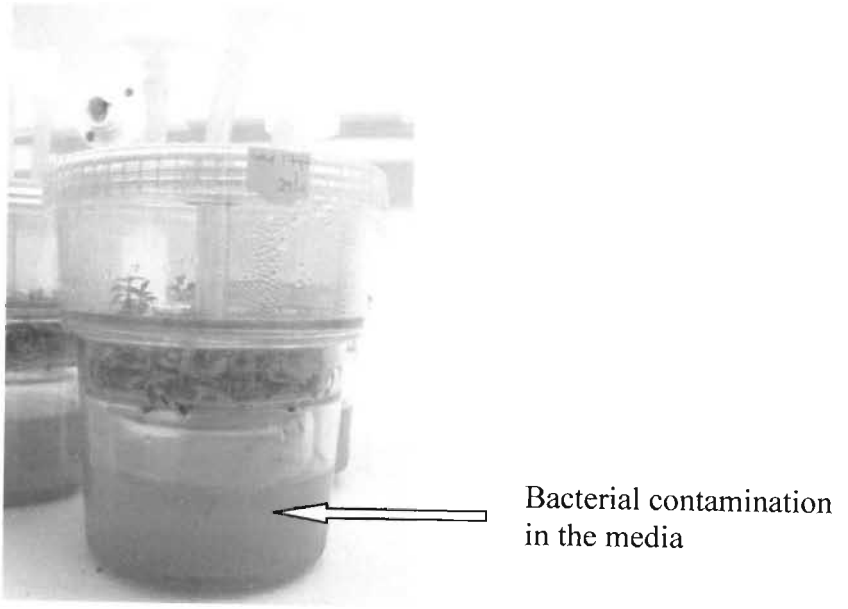


Figure 2.2. Bacterial contamination in the RITA® vessels

With the curative method, the fungicides were placed into the vessels containing visibly clean shoots (no visible fungal hyphae seen on the shoots), which were obtained from an infected

vessel. Newly initiated contaminant free shoots were used and the fungicides were placed into the vessels with the media as a preventative method for fungal contamination. The effects of the different fungicides on the shoots and the effectiveness of the fungicide as a curative or preventative method and its effect on plant growth can be seen in Table 2.6.

Table 2.6. Effectiveness of different fungicide treatments at different concentrations used in the RITA® vessels as curatives and preventatives on various *Eucalyptus* clones

Fungicides	**Clone**	**Concentration**	**Curative/ Preventative**	**Effectiveness on contamination**	**Effect on shoots**
Benlate®	GN107 GN108 NH58	0.1 $g.l^{-1}$ 0.5 $g.l^{-1}$ 1 $g.l^{-1}$	Curative	Fungal contamination	Shoots started to turn brown on all concentrations
		0.1 $g.l^{-1}$ 0.5 $g.l^{-1}$ 1 $g.l^{-1}$	Preventative	No contamination	Death of shoots occurred when left in the different concentrations for more than seven days
Bravo®	GN107 GN108 GN121 TAG31	0.5 $ml.l^{-1}$ 0.8 $ml.l^{-1}$ 1 $ml.l^{-1}$	Curative	Fungal contamination	No effect
		0.5 $ml.l^{-1}$ 0.8 $ml.l^{-1}$ 1 $ml.l^{-1}$	Preventative	No fungal contamination but bacterial contamination became a problem on all concentrations	No effect
Sporgon®	GN107 GN108 TAG31	0.1 $g.l^{-1}$ 1 $g.l^{-1}$	Curative	Fungal contamination	No effect
		0.1 $g.l^{-1}$ 1 $g.l^{-1}$	Preventative	No fungal contamination an out-break of bacterial contamination especially on the high concentration	Shoots suffered if left too long in this media
Sporekill®	GN107 GN108 NH58 TAG31	1 $ml.l^{-1}$ 2 $ml.l^{-1}$ 5 $ml.l^{-1}$	Preventative	Some fungal and bacterial contamination still occurred on all concentrations	No effect
PPM®	GN108	1 $ml.l^{-1}$ 2 $ml.l^{-1}$ 5 $ml.l^{-1}$	Curative	Contamination Fungal contamination Bacterial contamination	No effect Shoots died Shoots died
		1 $ml.l^{-1}$ 2 $ml.l^{-1}$ 5 $ml.l^{-1}$	Preventative		Shoots died on all concentrations
Puragene®	GN108 NH58 TAG31	0.05 $ml.l^{-1}$ 0.1 $ml.l^{-1}$	Curative		Plants died within two days
		0.05 $ml.l^{-1}$ 0.1 $ml.l^{-1}$	Preventative		Plants died within two days

The fungicides used as curatives were not effective as fungal contamination persisted in all the treatments. Benlate®, PPM® and Puragene® caused death of the shoots at the various concentrations on the different clones (Table 2.6). When used as a preventative (fungicides added to the medium to prevent fungal contamination), PPM® and Puragene® caused the shoots to die. This is in contrast with the findings of FULLER & PIZZEY (2001) who did not find PPM (1 $ml.l^{-1}$) to have any phytotoxic effect on *Brassica* and were successful in eliminating contaminants. Benlate® worked as a preventative but it should be used for two to three days only as necrosis of the shoots occurred after seven days. Benlate® has been used on many crops; it is a systemic fungicide and appears to act by interfering with the microtubules of the fungi (SHIELDS, ROBINSON & ANSLOW, 1984). Bravo® and Sporgon® eliminated the fungal contamination but there was a flush of bacterial contamination. Bravo® is thought to react with the thiol groups of cell constituents, which causes its biological activity (HASSALL, 1990). WATT *et al.* (1996) found that Benlate® and Bravo® significantly inhibited survival, multiplication and growth of *Eucalyptus grandis*, and that the phytotoxic effects persisted after they were planted in the soil. In conclusion, the use of fungicides as curatives and preventatives was not very successful. It is recommended, therefore that the vessels with fungal contamination be discarded rather than attempting to rescue the shoots.

Antibiotics were then tested on the cultures to determine if they could be employed as preventatives or curatives of bacterial contamination. Bacterial contamination occurred after three to five transfers in some *Eucalyptus* clones, both on the semi-solid and the liquid media thus indicating that some *Eucalyptus* clones have endogenous bacteria. The results of the uses of the different antibiotics are shown in Table 2.7.

When Rifampicin was used with the sponge supports in the RITA® vessels there was death of the plants as the sponge disintegrated and resulted in a toxic effect to the shoots. The antibiotic cocktail as a curative did not eliminate the bacterial contamination, but merely reduced it. Combinations of antibiotics can be successful but in many cases the antibiotics are chemically incompatible and may simply neutralize the effect of each other (FALKINER, 2000). Rifampicin at 0.1 $g.l^{-1}$ as a curative and as a preventative was effective in eliminating the bacterial contamination (Table 2.7). CORNU & MICHEL (1987) found that there was great variation between clones of *Prunus avium* L. (wild cherry) in the susceptibility to antibiotics. With the different *Eucalyptus* clones this was not found to be the case, although some of the

clones did have greater endogenous bacterial infections than other clones. NH58 and GU180 had more bacterial contamination than the other clones tested and, with the use of 0.1 $g.l^{-1}$ Rifampicin, the bacteria were inhibited but placement of these clones back onto antibiotic free media gave a flush of bacterial contamination.

Table 2.7. Effectiveness of different antibiotic treatments used as curatives and preventatives at different concentrations in the RITA® vessels on different clones

Antibiotics	Clone	Concentration	Curative /Preventative	Effectiveness on contamination	Effect on shoots
Rifampicin with sponges in the vessels.	GN121 GN108 GN107 NH58 TAG76	0.01 $g.l^{-1}$ 0.02 $g.l^{-1}$ 0.1 $g.l^{-1}$	Curative	Creamy coloured bacteria remained	Death of plants due to sponge disintegration
		0.01 $g.l^{-1}$ 0.02 $g.l^{-1}$ 0.1 $g.l^{-1}$	Preventative	Eliminated bacterial contamination but sponges disintegrated	Death of plants due to sponge disintegration
Rifampicin without the sponges	GU180 GU175 GN108	0.1 $g.l^{-1}$	Curative	Eliminated bacterial contamination.	No death of shoots – grew very well
		0.1 $g.l^{-1}$	Preventative	Eliminated bacterial contamination	No death of shoots – grew very well
Antibiotic cocktail	GN108 GN107 NH58 TAG31	Zinaceff 0.5 $ml.l^{-1}$ Novocillin 0.34 $ml.l^{-1}$ Novostrep 0.3 $ml.l^{-1}$ Claforan 0.2 $ml.l^{-1}$ Garamycin 0.2 $ml.l^{-1}$	Curative and Preventative	Reduced contamination but did not eliminate the bacterial contamination	No effect

As observed in this study, and reported by others, it is extremely difficult to eliminate bacterial and fungal pathogens from established plant tissue cultures using antibiotics, fungicides and other anti-microbial agents. In many cases, treatments only inhibit the contaminants and low levels of contamination persists (LEIFERT, 2000). This was certainly the case with most of the antibiotics and fungicides tested for elimination of contamination in the *Eucalyptus* culture. Microbial contamination can cause a decrease of the plantlet multiplication rates, a failure to root *in vitro* and can lower the plant survival rate during acclimatization (COOKE *et al.* 1992). In the present study with the use of Rifampicin bacterial contamination was contained to some extent thus allowing the plants to grow and develop without a detrimental effect. However, fungal contamination proved to be very difficult to eliminate and it seems that it is best to discard any vessels that have the smallest signs of fungal infection.

2.4. Conclusion

Obtaining contaminant free shoots in the RITA® system was achieved by using a two step approach of culturing on the semi-solid medium and 0.1 $g.l^{-1}$ Rifampicin added to liquid MS as a pre-treatment with visual selection of contaminant free plants, prior to initiation into the RITA® vessel. This was appropriate for all the *Eucalyptus* clones tested. When contamination occurred at the different stages of transfers, losses were far higher in the RITA® system compared with that of the semi-solid system, therefore it was important to maintain contaminant free cultures. It seemed that fungal contamination which occurred after establishment could not be eliminated and vessels that showed signs of contamination had to be discarded. Benlate® could be used as a preventative but the shoots had to be transferred within seven days otherwise the fungicide became toxic to them. A few of the fungicides tested led to bacterial flushes in the vessels. Rifampicin could be used as a curative to eliminate bacterial contamination. It could also be used as a preventative but this was not advisable as resistance to the antibiotic could occur.

Chapter 3. Multiplication

3.1. Introduction

Micropropagation is currently applied to a large number of forestry and agricultural species. The technique is costly due to intensive manipulation of the various phases (BERTHOULY & ETIENNE, 2002; PAEK & HAHN, 2002; ZIV, 2002). A great deal of time has been spent optimizing the media constituents and plant growth regulators for *in vitro* protocols of plants, while the features of the culture vessels have been arbitrarily selected (CASSELLS, 2000). However it has now been recognized that both the vessel and the atmosphere created within the vessel, have a significant role to play in relation to the micro plant quality. Bioreactors can provide a rapid and efficient plant propagation system but are not yet fully exploited commercially (ZIV, 2002). For optimal plant production by micropropagation in bioreactors it is essential to understand plant responses to micro-environmental signals and by manipulation of specific culture conditions, to control the morphogenesis of plants in liquid culture (ZIV, 2002). Temporary immersion systems have been described for a wide range of tropical crops, e.g. *Ananas comosus, Camellia sinensis, Citrus deliciosa, Coffea sp, Hevea brasiliensis, Manihot esculenta, Musa sp, Phalaenopsis* and *Solanum* (GONZÁLEZ, 2002), and they have been found to generally improve plant material quality. Furthermore, increased shoot vigour can be achieved and hyperhydricity, which seriously affects cultures in liquid media, is eliminated with these systems (BERTHOULY & ETIENNE, 2002).

There are many issues that need to be addressed in the development of a protocol for the commercial propagation of a particular plant (WALKER, 1995). According to WALKER (1995) when starting with the temporary immersion system in the multiplication stage where the goal is to increase multiplication some of the basic issues are:

- number of shoots to be placed in each vessel
- length of time spent in the vessels
- length of submersion and rest times
- media composition for the best multiplication

- size and type of vessel needed
- number of shoots to place in the vessel at the beginning of each multiplication cycle
- number of days per multiplication cycle
- temperature and light cycles required
- whether the liquid temporary immersion system is better than the conventional semi-solid system

Scientific inquiry can be used to determine the relationship between many of these variables and the multiplication ratio (WALKER, 1995). These types of issues were addressed in this study which aimed at developing a multiplication protocol to be used in commercial micropropagation of *Eucalyptus* clones using the temporary immersion bioreactor system (RITA®).

3.2. Materials and Methods

The choice of clones selected for use in these studies was based on availability of material.

3.2.1. Establishment of culture parameters

At all stages the plants were grown in growth rooms between 22-25 °C, at 16 hours light/eight hours dark photoperiod. Cool white flourescent lights illuminated the growth rooms (1 360 lux), unless otherwise stated. In the RITA® vessels 200-250 ml of media was used with 25 ml per jar used for the semi-solid system. The media was made to a pH of 5.8 and autoclaved at a pressure of 1Kpa at a temperature of 121 °C. An unequal cyclical timer (varied according to the experiment) controlled the flush and rest periods and a fish tank pump was used to push air through the system (Figure 3.1).

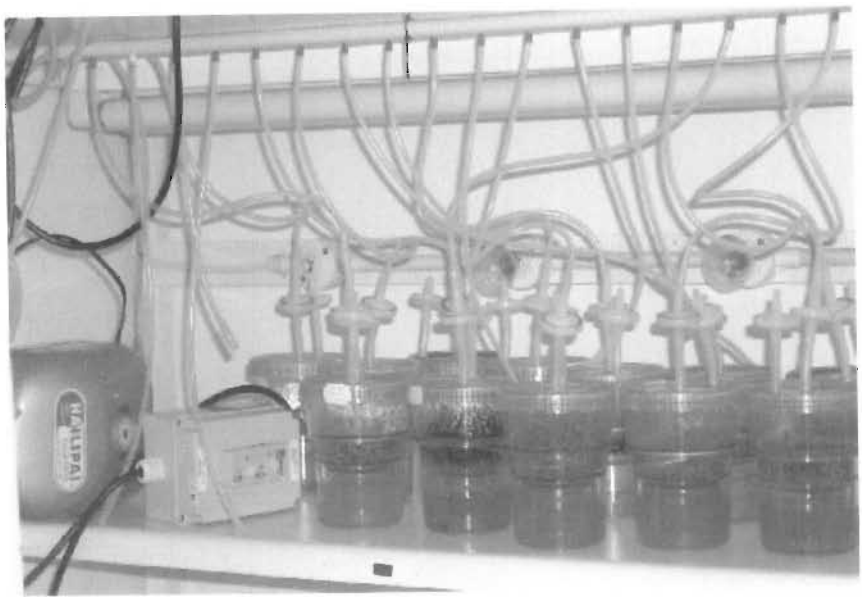


Figure 3.1. Fish tank pump (left) and timer that control the flush and rest periods are connected to the RITA® vessels

3.2.1.1. Effect of flush and rest times on multiplication

Thirty shoots of GN108 were placed into RITA® vessels (three vessels per treatment) containing M1 media (Appendix 2). Different flush times – where the plants are submerged in the media (30 sec, 1, 5 and 10 min) and rest times – where the plants are not covered with media (5, 10 and 20 min) were used. The multiplication was recorded after 14 days to determine which flush and rest times gave the highest multiplication.

3.2.1.2. Effect of different numbers of starting shoots per vessel and interval times on multiplication

To test the effect of different numbers of shoots per vessel, 50, 100 and 150 shoots were placed into RITA® vessels and flushed for 30 seconds (flush time found to be good for multiplication). Rest periods of 10, 20 and 30 minutes were used for the different numbers of shoots per vessel (50, 100 and 150). GN108, NH58 and TAG31 were the three clones used, as enough sterile explants were available. Shoots were placed into multiplication media (M1, Appendix 2), and shoot multiplication was recorded after 21 days. Multiplication is the number of shoots produced after a certain time

period divided by the initial starting number of shoots (this was used to calculate all multiplication rates throughout the study).

3.2.1.3. Effect of different media on multiplication

a. Variations of the standard multiplication media

Different variations of the multiplication media (M1- M5, Appendix 2) were tested using different clones (Table 3.1). The various multiplication media had different concentrations of sucrose, 25 $g.l^{-1}$ in M1and M2 and 20 $g.l^{-1}$ in M3-M5. They also had different concentrations of cytokinins and auxins (Appendix 2). Fifty shoots per vessel with a flush time of 30 seconds and a rest of 10 minutes were used. Multiplication numbers were recorded.

Table 3.1. Multiplication media used for select *Eucalyptus* clones

Media (Appendix 2)	Clones tested
M1	GU175, GU177, GU178, GU180, GN107, GN108, NH58
M2	TAG31, GN107, GN108, NH58
M3	GU175, GU177, GU178, GU180, GN107, GN108, NH58
M4	GU175, GU177, GU178, GU180, GN107, GN108, NH58
M5	GU175, GU177, GU178, GU180, GN107, GN108, NH58

b. Effect of starting media on multiplication at successive cycles

As it was observed during the elongation trials that return of the shoots to multiplication medium (M1) from elongation medium (E1) (Appendix 2) enhanced multiplication, this was investigated further. Fifty shoots of GN108 were placed onto different media for 14 days (Table 3.2, Appendix 2). The starting medium was used as the treatment medium. Thereafter the shoots were placed on two rotations of multiplication media (M1, Appendix 2) for 14 days at each rotation. Multiplication was recorded to determine the effect the starting media had on the multiplication numbers at each stage.

Table 3.2. Sequences of media (Appendix 2) used to determine the multiplication for GN108 (shoots were 14 days in each medium).

Treatment	Starting media	2nd rotation	3rd rotation
1	M1	M1	M1
2	M2	M1	M1
3	E1	M1	M1
4	E2	M1	M1
5	MS	M1	M1
6	½ MS	M1	M1

3.2.2. Comparison of multiplication in the RITA® *vs.* the semi-solid system

a. Comparison of multiplication rates of five clones over a 14 to 28 day period

Five clones were grown over a 14 to 28 day period with three replications for each clone. Fifty shoots were placed into RITA® vessels with 250 ml of liquid M1 medium per vessel and 50 shoots (eight per jar) were placed into jars with 25 ml per jar of semi-solid M1 medium (Appendix 2). A flush time of 30 seconds every 10 minutes was used for the liquid system. Multiplication was recorded for both systems.

b. Comparison of multiplication of two cold-tolerant clones over successive multiplication cycles in the RITA® *vs.* the semi-solid system

NH58 was grown for 71 days and GN108 was grown for 105 days on M1 media (Appendix 2), with transfers every 14-21 days in the temporary immersion system and 20-30 days in the semi-solid system. The multiplication was recorded at each transfer.

3.2.3. Interaction of nutrient change within multiplication media over 21 days in the semi-solid and RITA® systems

Fifty shoots per RITA® vessel of GU177 with three replications per treatment were initiated in M1 liquid media. Fifty shoots were placed onto semi-solid media (seven shoots per jar) with three replications for each treatment. At day 0, 7, 14 and 21 the shoots were destructively harvested and

the multiplication and shoot size of plants from the two systems were recorded. At harvest on days 0, 7, 14 and 21 the electrical conductivity (EC) and pH of the used media was recorded for the semi-solid medium and the liquid medium. The plants grown on the respective media at each harvest together with the media on which they were grown was sent to the Agricultural Research Council (ARC) in Nelspruit for elemental analysis. The data was then analyzed to determine if there was a difference in uptake of the nutrients at different times in the cycle that could be giving the increased multiplication.

3.2.4. Data analysis

All data was analyzed statistically using multiple analysis of variance (ANOVA) and differences were compared using Duncan's multiple range test.

3.3. Results and Discussion

3.3.1. Establishment of flush/rest times and optimum number of shoots to use per vessel

Oxygen and carbon dioxide are principal substrates or products of aerobic respiration and photosynthesis and can affect the most basic metabolic pathways in a plant cell. As tissues grow, the risks of inadequate ventilation also grows and the build up of ethylene and other gases can cause asphyxiation of plant material (JACKSON, 2002). The temporary immersion system provides a highly aerobic system for plant growth, as there is forced ventilation through the vessel lid. However the immersion time, i.e. duration or frequency is the most decisive parameter for system efficiency (ALVARD *et al.* 1993; BERTHOULY & ETIENNE, 2002). Different flush and rest times were used to determine the effect they had on multiplication. Thirty shoots per vessel were used as, this was deemed to be an appropriate number to place into the vessels.

It was found that the five minute rest period gave significantly lower multiplication i.e. from 2.1 times (30 sec flush) to 1.5 times at 10 minutes flush (Figure 3.2.).

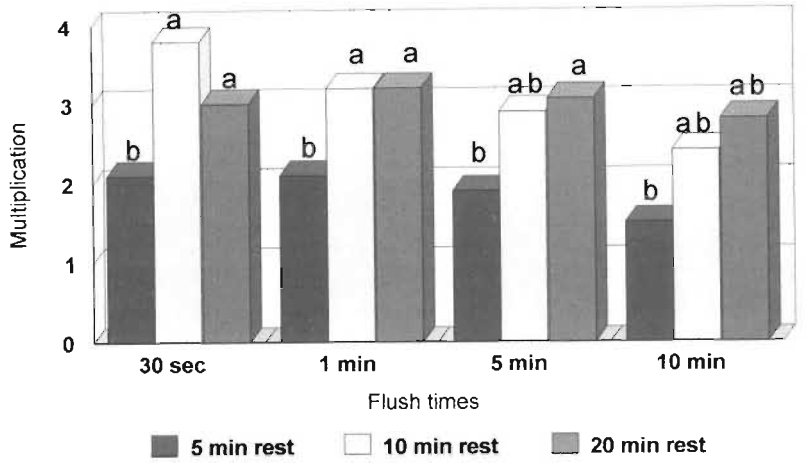


Figure 3.2. Multiplication of the shoots of GN108 placed in the RITA® vessels at different flush times and rest times ($p < 0.01$)

At the 10 minute flush time with a five minute rest period the *Eucalyptus* shoots became brittle and hyperhydricity occurred. This was probably due to the flush intervals being too frequent thus causing the same problems that occurred when using a continuous immersion system (as found in the pilot study, Appendix 1). JACKSON (2002) stated that an aqueous cover interferes strongly with gas exchange to the outer tissue or cell surface since gas diffusion rates are approximately 10000 times slower in water than in air. This impact is increased with the depth of the aqueous cover or the inclusion of solid matrices such as agar. Thus, by total submersion or submersion of the plants too frequently for long periods, gaseous exchange for photosynthesis and respiration was reduced even if there was dissolved oxygen and carbon dioxide in the liquid. When the vessel was flushing the plants were totally submerged in the nutrients (Figure 3.3) and when the rest period occurred there was no submersion of the plants at all (Figure 3.4). If the frequency of the rest period was too short and the time the plants were submerged was too long, problems occurred. This could have been the cause of the hyperhydricity and brittleness of the shoots at the 10 minute flush time and five minute rest time. BERTHOULY & ETIENNE (2002) reduced hyperhydricity by adjusting the immersion times of the RITA® system and allowing better gaseous exchange to occur. FUJIWARA & KOZAI (1995) found that increasing the number of air exchanges avoided shoots becoming hyperhydric with long-term continuous liquid cultures.

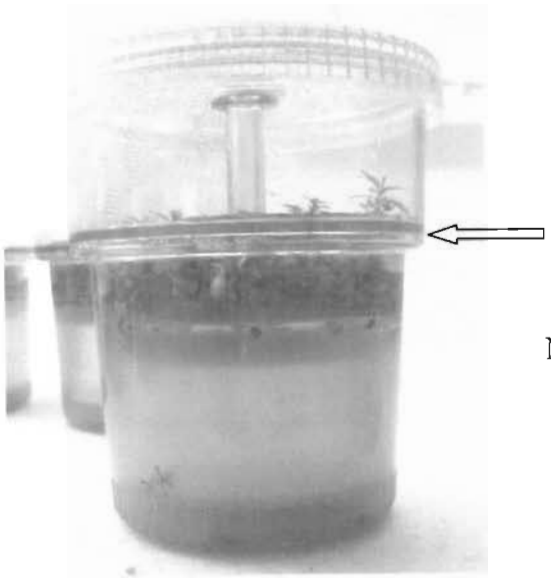


Figure 3.3. Total submersion of the shoots by the nutrients at the flush time

Nutrient level

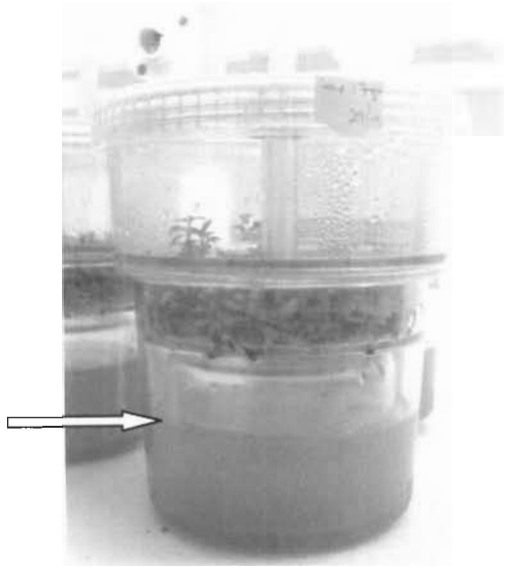


Figure 3.4. Plants not submerged by the nutrients at the rest period

The 10 minute flush time caused a reduction in the multiplication (Figure 3.2). However, with the increased rest time between the flushes (10 and 20 minutes rest time) there was an increase in multiplication. These results indicated that there was a relationship between the length of flush and rest time – with an increase in flush time the shoots required an increase in the rest period (Figure 3.2). One minute flush time at 10 and 20 minute rest time and five minute flush time at 20 minute rest time gave high (3.2x, 3.2x and 3x) multiplication.

For the *Eucalyptus* shoots, a flush time of 30 seconds with a rest period of 10 minutes gave the highest multiplication (3.8x). Different plants require different flush and rest times for optimal multiplication and many researchers found that the immersion time affected the plant growth rate. PREIL & HEMPFLING (2002) found with *Phalaenopsis* that the effect of immersion frequency affected the plant growth rates and eight immersions for 10 minutes per day were applied which gave optimal multiplication. ALVARD *et al.* (1993) found that 20 minutes every two hours was optimal for bananas. By controlling the immersion cycles, AKULA *et al.* (2000) achieved a more

consistent and synchronized multiplication and embryo development of tea. They used one minute immersions every six hours to obtain a 24 fold increase. If they decreased the number of immersions to nine or 12 hours the multiplication decreased to 15 fold and 10 fold respectively and by increasing the immersions to every three hours the multiplication also reduced to 10 fold. DAMIANO *et al.* (2000) reported that immersion time of 15 minutes for wild pear was too short and two hours was too long. However with *Eucalyptus* a submersion for 15 minutes would have been too long. MARTRE *et al.* (2001) reported that the immersed stage induced a substantial oxidative stress on *Hevea brasiliensis* callus. This oxidative stress could explain the time variations of the multiplication at the different immersion times. The immersion time intervals play a decisive role in influencing the multiplication rates for different species as this factor affects nutrient supply and composition of the internal atmosphere in the culture vessel (JIMÉNEZ *et al.* 2000). Temporary immersion for 30 seconds every 10 minutes was most beneficial for *Eucalyptus* (GN108) multiplication.

It became evident from the study on the interval and submersion times that the number of shoots in the vessels had an effect on the time required between submersions and, contrary to previous assumptions, 30 shoots per vessel may not have been the optimal number for maximum multiplication. Consequently, for this reason 50, 100 and 150 shoots were tested at different rest times to test this further. The results showed that there was a significant difference in multiplication of plants as a result of the number of starting shoots, with 50 shoots per vessel giving the best multiplication for all three rest times tested (Figure 3.5). Starting with 50 shoots per vessel the multiplication of the three clones tested were significantly higher using a 30 second flush every 10 or 20 minutes (2.74, 2.66x respectively). The rest time of 30 minutes gave the lowest multiplication (1.3x) using 100 and 150 shoots per vessel. With more shoots per vessel a decrease in the length of time between flushes was required as more shoots per vessel led to a decrease in the availability of nutrients. More shoots led to the depletion of nutrients at a faster rate.

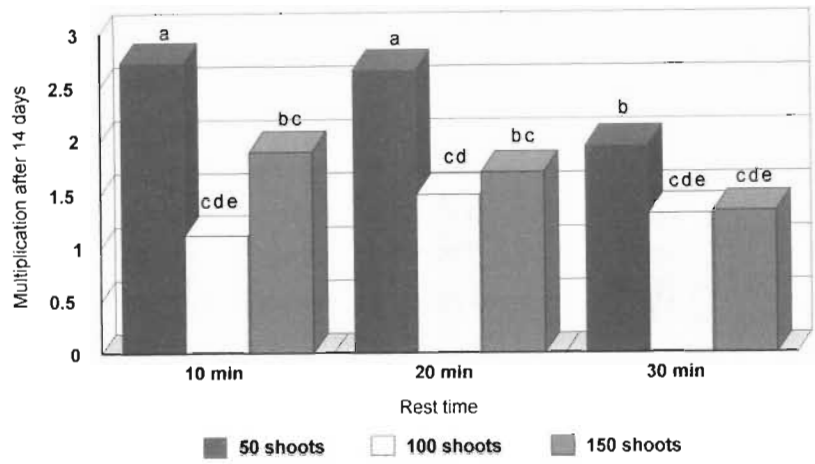


Figure 3.5. Effect of different rest times (10, 20 and 30 min) and shoot numbers per vessel on multiplication rates after 14 days for three clones (a 30 second flush time was used) ($p< 0.01$)

Optimizing the volume of nutrient medium and the volume of the container also substantially improve efficacy, especially shoot proliferation (BERTHOULY & ETIENNE, 2002). MONETTE (1986) showed that the ratio of medium volume to vessel size and explant density affected growth and improved growth was obtained with larger vessels. The RITA® vessels are a set size, but it was important to use the correct volume of medium in the vessels. An amount of 100 ml of medium per container was insufficient, as there was not enough medium to submerge the plants effectively when flushing. 200-250 ml covered the plants sufficiently with the medium at the flushing time (Figure 3.3). A volume higher than 250 ml caused the plants to float and damage occurred. ESCALONA *et al.* (1999) achieved a high multiplication with pineapple when a volume of 200 ml was used in the temporary immersion system and volumes higher than 200 ml led to a decrease in multiplication.

3.3.2. Media for increased multiplication in the RITA® system

a. Media containing different plant growth regulators and sucrose concentrations

For production of *Eucalyptus* in a commercial laboratory utilizing the semi-solid system, four main multiplication media have been formulated and are used at different times for different clones. These have variations of sucrose and plant growth regulator concentrations (M1, M3, M4 and M5, Appendix 2). The standard medium used in the semi-solid system was M1 (Appendix 2). However

differences in the multiplication of the various clones differ with the use of various media. These media were tested on different clones in the RITA® system to determine if there was a variance in multiplication between the clones on the different media. It was also necessary to find a standard medium that could be used for all clone types.

A significant difference was found in the multiplication between the clones and between the media when the averages were taken (clones average multiplication and media multiplication, Table 3.3). M2 gave the lowest multiplication for all the clones tested. This medium had half strength MS nutrients indicating that full strength was needed at the multiplication stage. The highest average multiplication (5.63x) of the clones was with M1, a medium with 25 $g.l^{-1}$ sucrose and 0.2 $mg.l^{-1}$ BA and 0.01 $mg.l^{-1}$ NAA. However, with M3 that had the same concentration of plant growth regulators but 20 g.l-1 sucrose a multiplication of 4.89x was obtained across the clones, indicating that improved multiplication was achieved on higher sucrose concentration. On M5 medium where the concentration of plant growth regulators was higher (0.5$mg.l^{-1}$ BA and 0.2 $mg.l^{-1}$ NAA) and the sucrose was 20 $g.l^{-1}$, the average multiplication was 5.15x. However, on this medium the plants all tended to be very small in size ranging from 0.5-2 cm (Table 3.4), whereas those on M1 media were bigger (0.5-7 cm) darker green and healthy (Figure 3.6). The medium (M4) with equal concentrations of BA and NAA (0.5 $mg.l^{-1}$) gave a lower multiplication of 4.2x, and GN107 had callus formation on the shoots on this media.

Table 3.3. Effect of different media sequences on multiplication (x) for select *Eucalyptus* clones ($p<0.01$)

Clone	Multiplication (x)					
	M1	M2	M3	M4	M5	Clones average
GU175	5.4 h		5.5 gh	5.2 hi	4.8 i	4.76 e
GU177	8.5 a		7.9 b	6.2 ef	8.2 a	7.31 a
GU178	7.2 c	1.2 q	7.3 cd	6.5 de	7.1 d	5.91 b
GU180	4.3 jk	2.5 no	4.2 k	2.6 no	3.5 l	3.42 f
TAG31	6.4 e	4.3 jk	5.5 gh	4.2 k	5.9 fg	5.26 d
GN107	2.4 o	1.2 q	1.8 p	1.7 p	1.5 pq	1.72 h
GN108	7.4 c	3.1 lm	4.3 jk	4.7 ij	6.9 d	5.28 c
NH58	3.2 lm	1.5 pq	2.6 no	2.5 no	3.3 lm	2.62 g
Average multiplication of the media	5.63 a	2.81 e	4.89 c	4.2 d	5.15 b	

Figure 3.6. Healthy, large dark green shoots on M1 medium

Each clone had different multiplication rates on the media tested, which suggests a clonal specificity to different media. Though M1 medium can be considered "universal" as it mostly gave the best multiplication and good quality shoots (Figure 3.6), some of the clones had improved multiplication on other media. GU175 had the best multiplication on M3 medium (5.5x) and on M1 the shoots were hyperhydric (Table 3.4). GU177 had high multiplication across all the media although the best plant quality (healthy big shoots) was achieved on M1 medium. GU178 had high multiplication on all media except for M2. However, M1and M2 gave hyperhydric shoots and M3 and M4 had the best quality shoots. GU180 had lower multiplication (average of 3.42x) compared with the other sub-tropical clones (averages between 4.76 and 7.31x). The best quality plants were obtained on M3 media for GU180 and M2 gave hyperhydric shoots. TAG31 had the best multiplication on M1. Both M1 and M3 gave good quality plants. GN107 and NH58 had the lowest multiplication across the media (1.72 and 2.62x respectively). These are cold-tolerant clones and, on the whole, are known to be slow growers when cultured on the conventional semi-solid system however GN108

had a high multiplication across the media (5.28x). For the three cold-tolerant clones M1 media achieved the highest multiplication.

Table 3.4. Effect of media (Appendix 2) on the various clones
+++ – Healthy big shoots, ++ – Good shoots, + – Small shoots,
C – Callusing a little, - – Hyperhydric shoots

	Media				
Clone	**M1**	**M2**	**M3**	**M4**	**M5**
GU175	-		++	++	+
GU177	+++		++	++	+
GU178	-	-	++	++	+
GU180	++	-	+++	++	+
TAG31	+++	++	+++	++	+
GN107	+++	++	++	C	+
GN108	+++	++	++	++	+
NH58	+++	++	++	++	+

The lower growth rates observed on M1 media may be attributed to the carbon source being insufficient to sustain the multiplication. M4 and M5 gave lower multiplication across most of the clones. M4 had higher BA and NAA concentrations and most clones multiplied well and the shoots were of a good quality although GN107 callused on this media. Growth on M5 resulted in good multiplication but the shoots were very small.

It was evident that some of the media caused hyperhydricity in some clones but when they were placed on different media this was eliminated. PHAN (1991) used semi-solid media in jars and WELANDER, ZHU & LI (2002) used liquid media in the RITA® system for the propagation of apple and both found that high concentrations of BA resulted in high hyperhydricity. This was not the case with *Eucalyptus* as M4 and M5 have higher concentrations of BA and the hyperhydricity found in GU 175, GU178 and GU180 was eliminated.

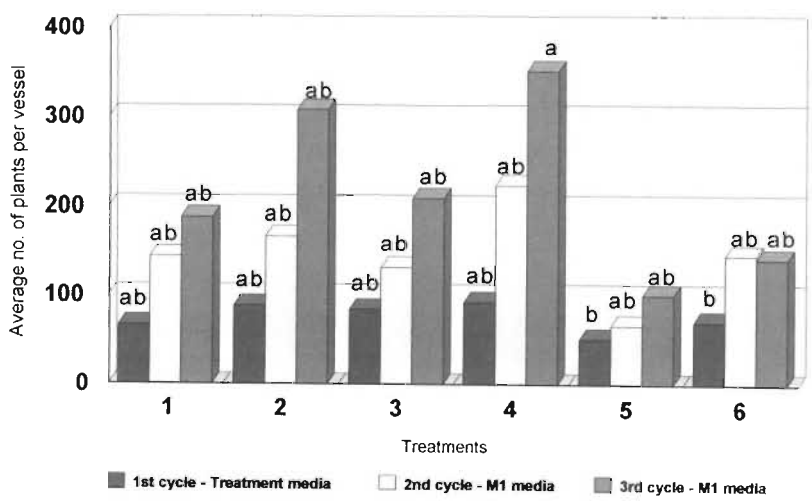
For the *Eucalyptus* clones tested changing the sucrose and plant growth regulators caused changes in plant size and multiplication. The different types of shoots produced with these media can be seen in Table 3.4. Mostly M1, M3 and M4 media produced good healthy shoots. In the RITA® system M1 media can be used as a standard multiplication media across the clones and other media may be used when needed to eliminate hyperhydricity, which may occur in the RITA® system with a few of

the clones. KOZAI (1988) reported that the growth of plantlets *in vitro* could be promoted by carbon dioxide enrichment under high photosynthetic photon flux, without any sucrose in the medium. This would allow for little bacterial or fungal contamination in the vessel during the propagation. If plantlets become vigorous on sucrose free medium then no acclimatization may be required in many cases.

b. Effect of different starting media on multiplication after two cycles

From a pilot study it was found that starting media had an influence on the subsequent multiplication. That study showed that starting with E2 media (Appendix 2) then transferring to M1 and a further rotation of M1 gave approximately twice the multiplication compared with the control (three rotations of M1 media). Using E1 media and then transferring to M1 medium gave four times the multiplication. Some rooting occurred on both E1and E2 media. Different media were used as starting media and subsequently two cycles of M1 were used and the multiplication was recorded for the time on each of the different media treatments.

Placement of 50 starting shoots per treatment on the various treatment media gave low multiplication numbers – blue bars (Figure 3.7). Thereafter the multiplication numbers increased.



Key: Treatments

	Blue	to Yellow	to Green
1.	M1	to M1	to M1
2.	M2	to M1	to M1
3.	E1	to M1	to M1
4.	E2	to M1	to M1
5.	MS	to M1	to M1
6.	½ MS	to M1	to M1

Figure 3.7. Total multiplication over three cycles (14 days for each cycle). The green bar indicates the final number of shoots accumulated at the end of the third cycle (average from three vessels starting with 50 shoots at the beginning of the cycles)

Statistically there was little difference in multiplication numbers between the treatments. However there was a difference in the multiplication numbers by the third cycle (green bar, Figure 3.7). Shoots on treatment 5 and 6 gave very low numbers (averages of 100 and 141 shoots produced respectively after the total of 42 days in culture). However the size of the plants increased from an average of two centimeters to between five and seven centimeters. These two media gave large healthy plants and should be used prior to rooting to increase the size of the shoots. Treatments 5 and 6 where ½ MS and MS were used as the starting media, did not have plant growth regulators. Thereafter the two cycles of M1 media had plant growth regulators. Treatment 6 where ½ MS media was used once (Appendix 2) and then transferred onto M1 media with the full concentration of nutrients gave a multiplication of 2.1x (Table 3.5). Placing the shoots under nutrient stress for a cycle and then placement onto high nutrients stimulates multiplication (as in treatment 2, 4 and 6. Table 3.5). Treatment 4 gave the greatest number of shoots over the three cycles (an average of 350 shoots). At the first cycle on the treatment media multiplication was the highest of all the treatments (1.9x) and improved further once placed onto full nutrients. The multiplication between the first cycle and second cycle was 2.4x, after which it dropped to 1.6x. Generally the multiplication between the first cycle (treatment cycle) and the second cycle (M1 media) were the highest and dropped on the third cycle (M1 media). Treatment 1 (the control, placement of shoots onto M1 for the three cycles) did not achieve the highest numbers of shoots or the highest multiplication between the cycles.

Table 3.5. Multiplication (number of shoots at the start of each cycle/number of shoots at the end of the cycle) over three cycles with the first multiplication being the treatment cycle (14 days for each cycle)

Treatment	Treatment media	1st cycle Treatment media	2nd cycle M1 media	3rd cycle M1 media	Average multiplication rate over 42 days (Final numbers /starting number of shoots)
1	M1	1.3	2.1	1.3	3.76
2	M2	1.7	1.9	1.84	6.14
3	E1	1.7	1.6	1.6	4.16
4	E2	1.9	2.4	1.6	7
5	MS	1.03	1.3	1.5	2
6	½ MS	1.4	2.1	0.9	2.8

Treatments 2 and 4 gave the highest average multiplication for the three cycles (6.24 and 7x respectively). Both of these media had half strength nutrients in the first cycle and contained plant growth regulators (Appendix 2). Treatment 6 also had half strength nutrients, but did not have plant growth regulators. It gave a low average multiplication (2.8x).

Subsequent transfers using M1 media continuously resulted in smaller shoots, from 2-3cm to 0.5 cm by the end of the third cycle. It was an indication that continued use of this specific medium would not be recommended and the plants should be placed onto an elongation media to increase the shoot size and optimize multiplication. The use of E1 and E2 media induced some root development, thus should be considered as rooting media. AMIRI (2001) stated that the mineral uptake is proportionate to the mineral availability, which is influenced by mineral diffusion, and mineral diffusion is the dominant process in mineral availability. The rate of mineral uptake was assumed to be proportionate to increased growth. With the use of M2, E2 and ½ MS media the rate of growth was very low as there was half the amount of nutrients available for diffusion and as soon as the plants were moved to M1 medium with higher nutrients, growth occurred. There were more minerals to diffuse so growth increased. To obtain optimal multiplication and large plants for *Eucalyptus* it would be advisable to use M2, E2 or MS media in alternation with M1 medium to obtain optimal multiplication and larger plants.

3.3.3. Comparison of multiplication in the RITA® *vs.* the semi-solid system

a. Comparison of multiplication and determination of the optimal cycle time for the two systems

Average multiplication of shoots for three sub-tropical clones and two cold-tolerant clones were calculated for the semi-solid (for 28 days) and RITA® system (for 14 days). All clones had different multiplication (Table 3.6). The clones all multiplied faster in the RITA® system compared with plants in the semi-solid system (Table 3.6).

Table 3.6. Multiplication of shoots (from 100 starting shoots) in the semi-solid system (28 days) and RITA® system (14 days) of different *Eucalyptus* clones and average multiplication for the sub-tropical and cold-tolerant clones (s.d. indicates the standard deviation of the multiplication)

Clone	Semi-solid (28 days) starting with 100 shoots	Liquid (14 days) starting with 100 shoots
GU177	497	845
GU178	376	722
TAG31	526	637
Average multiplication for the subtropical clones	4.7 times (s.d. 0.78)	7.3 times (s.d. 1.05)
GN107	187	237
GN108	294	744
Average multiplication for the cold-tolerant clones	2.4 times (s.d. 0.54)	4.9 times (s.d. 2.45)

After 28 days in the semi-solid system, subtropical clones (GU177, GU178, TAG31) achieved a multiplication of 4.7x (s.d. 0.78) while in the RITA® system the same clones achieved 7.3x (s.d. 1.05) over a 14 day period. The shoots of cold-tolerant clones (GN107, GN108) multiplied by 2.4x (s.d. 0.54) in the semi-solid over 28 days and, a 4.9x (s.d. 2.45) in the RITA® system over 14 days. The optimum multiplication cycles in RITA® were between 14 and 21 days (Figure 3.8), whereas in the semi-solid system they were 25 to 28 days (Figure 3.9).

Day 0 Day 14

Figure 3.8. Fifty shoots in the RITA® system at the beginning of the cycle (left) and the multiplication which occurred in the vessels after 14 days (right)

Day 0 Day 28

Figure 3.9. Seven shoots per jar at the start of a cycle in the semi-solid system (left) and the multiplication that occurred in a jar after 28 days (right)

The shoots in the RITA® system began to deteriorate quickly and started to die if they were left longer than 21 days in the system. PREIL & HEMPFLING (2002) also found that with *Phalaenopsis* the media had to be changed at two week intervals as the four week intervals of media exchange resulted in a distinct reduction of propagation efficiency.

The vessel closure regulates the degree to which the physiochemical factors in the growth room impact on the micro-environment as the type of closure forms the interface between the inside and outside environments of the vessels (SMITH & SPOMER, 1995). The type of closure affects the gas composition in the vessels (JACKSON *et al.* 1991). With the semi-solid system a major barrier to tissue aeration is the enclosing of a vessel to prevent drying out and contamination. This vessel closure often results in asphyxiation (JACKSON, 2002). According to JACKSON (2002) forced ventilation allows the plants to become more photoautotrophic which enhances growth and the oxygen and carbon dioxide availability in the temporary immersion system allows aerobic respiration and photosynthesis to occur with no build up of ethylene in the vessels. KHAN, KOZAI, NGUYEN, KUBOTA & DHAWAN (2002) looked at growth and photosynthetic rates in *Eucalyptus tereticornis* Smith, and they found that photomixotrophic conditions gave the best multiplication rates of the plants (the plants were grown on agar with CO_2 forced into the system). The exchange

of gases in the RITA® system could be one of the factors leading to the increased growth rates observed. ZOYBAYED, ARMSTRONG & ARMSTRONG (2001) reported that sealing of culture vessels could seriously inhibit growth and development and could induce hyperhydricity and reduce the leaf chlorophyll content. They also found that in the light period CO_2 depletion occurred in the headspace of a sealed vessel but CO_2 increased with the improved efficiency of the ventilation. There was no ethylene accumulation when there was ventilation. Thus the differences in the multiplication numbers were due to the different physical environments that the plants were exposed to which led to changes in the chemical environment. The physical and chemical environments are interrelated, and with the ability to change the physical environment (vessel type, closure, headspace, gels and light) changes in the chemical environment occur (uptake of nutrients and biological pathways are changed).

KOKKO *et al.* (2002) grew different Aspen clones in the RITA® system and found that there was an increase in the multiplication numbers. The growth and multiplication in agar varied markedly between clones but when placed in the RITA® system the differences were less marked. Many of the comparisons between the different systems untaken by other researchers (Table 1.2) show that there is a marked increase in multiplication using a temporary immersion system.

b. Comparison of multiplication over several cycles

Two cold-tolerant *Eucalyptus* clones were then taken and multiplication was recorded over a series of transfers to determine if multiplication achieved in the semi-solid system could be comparable to those on the RITA® system over a long period of time. This also allowed for phenotypic observation of shoots in regard to size and colour changes over time in the two systems. From Table 3.7, it can be seen that the multiplication for the clones were different in the two systems (Figure 3.10). The RITA® system produced higher multiplication over time for both clones although it was slower for GN108.

Table 3.7. Multiplication in the RITA® and semi-solid systems for two cold-tolerant clones

** Plants were being rooted off at this stage as there were not enough vessels to place all the shoots back onto multiplication media so multiplication numbers were being affected slightly

	RITA®	Semi-solid		RITA®	Semi-solid
Days	**NH058**	**NH058**	**Days**	**GN108**	**GN108**
0	60	60	0	57	60
14	196		18	139	72
21	330	138	41	228	136
33	**960		55	**307	204
52	**1796	358	83	**503	408
71	**3084	859	105	**988	734

Figure 3.10. Differences in shoot size and multiplication in the RITA® system (left) and the semi-solid system (right)

The results indicate that there were clonal differences and conditions for each of the two clones that were placed into the RITA® vessels. There was a great difference in size, colour, leaf expansion and stem quality of the plants (Table 3.8, Figure 3.11). The *Euclayptus* shoots produced in the RITA® system were superior in quality to those produced on the semi-solid system. KOKKO *et al.* (2002) had similar findings with *Aspen* shoots grown in the RITA® system compared with that on agar. Diffusion is the dominant process in mineral availability *in vitro.* As previously stated low rates of diffusion of minerals through a gelled medium occur, and therefore a low uptake rate and efficiency

occurs (AMIRI, 2001). In the liquid medium mineral diffusion can readily occur giving better quality plants with higher growth rates.

Table 3.8. Phenotypic differences of GN108 shoots from the semi-solid and RITA® systems
++++ very good +++ good + poor

	Semi-solid	**RITA®**
Height	2.5 cm	4 cm
Colour	Pale green	Dark green
Leaf expansion	+	+++
Multiplication	+	++++

Figure 3.11. Phenotypic differences of the shoots produced from the two systems

It is vitally important to obtain good quality plants in the multiplication stage as the quality of the plant produced here influences the rooting and acclimatization that occur at later stages. The physiological status of the tissue cultured plants have a tremendous impact on their subsequent survival and rooting at acclimatization in the greenhouse (DEBERGH, TOPOONYANONT, VAN HUYLENBROECK, MOREIRA DA SILVA & OYAERT, 2000).

GONZÁLEZ (2002) stated that the RITA® system was a suitable tool for research, laboratory protocols standardization and small scale-up but for commercial application larger vessels are usually needed. GONZÁLEZ (2002) found that out of seven species commercially propagated, six are performed in twin flasks TIS with volumes ranging form 5-10 l glass or poly carbonate vessels. On the other hand KOKKO *et al.* (2002) are developing protocols in the RITA® system for the commercial propagation of *Aspen* for the forestry industry. From the results obtain in the present study the findings are that a protocol for the commercial application of the RITA® system for the production of *Eucalyptus* can be achieved and it is evident that the RITA® system can be utilized for commercial production of *Eucalyptus*. The multiplication and the plant quality are superior with the RITA® system compared with those from the semi-solid system. It is unknown what the plant quality would be if a larger vessel was used for the production of *Eucalyptus*. With the use of a larger container there is a correspondingly greater risk of losses from contamination (Chapter 2).

3.3.4. Interaction of nutrient change with multiplication over 21 days in the semi-solid and RITA® systems

Liquid culture media permits more efficient nutrient uptake (ESCALONA *et al.* 1999). AMIRI (2001) hypothesized that mineral uptake and hence growth is proportional to the initial concentration of minerals in the media. At intervals throughout the 21 days there were differences occurring in the uptake, which could be attributed to diffusion. There were also changes in shoot size, numbers and EC in the two systems at different times. When each individual nutrient was analyzed in some cases there was a statistical difference in the nutrients in the media and in the plants at the different time intervals (Figures 3.16-3.35).

Multiplication from 100 starting explants in both systems (RITA® - 50 per vessel and semi-solid – eight per jar) the RITA® system far exceeded the semi-solid system in multiplication. Shoot numbers in the RITA® system increased from 423 to 744 between day seven and day 14, and from 744 to 888 between day 14 and 21 (Figure 3.12). Between day 14 and 21 shoot elongation increased considerably, thus making it feasible to culture the shoots in RITA® for 21 days (Figure 3.13). The

multiplication slows and elongation occurs at this time. The semi-solid system gave smaller plants and multiplication was lower and plant numbers were achieved more slowly. There was a decrease in the shoot length of the plants at day 14 in the semi-solid system which could be due to the manner in which the shoots are excised from the main stem.

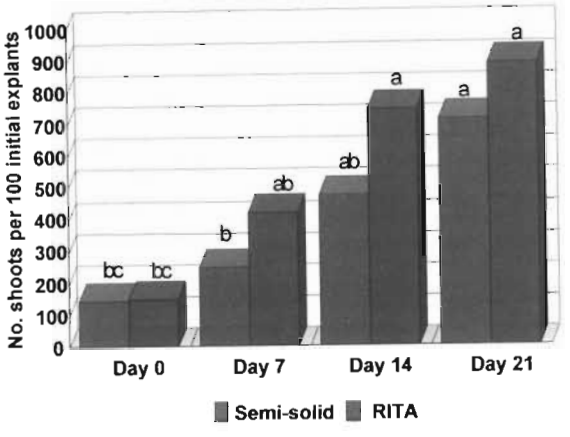


Figure 3.12. Multiplication in the RITA® and semi-solid systems (per 100 starting shoots) over 21 days ($p < 0.01$).

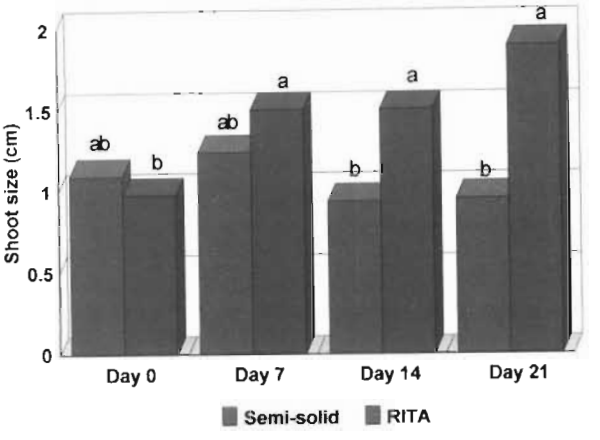


Figure 3.13. Shoot length (cm) in the RITA® and semi-solid systems over 21days (minimum of 100 shoots per system per time period) ($p< 0.01$)

The EC in the RITA® system was high at the beginning (5.5 μS) (Figure 3.14). It dropped steadily over the 21 day period with the greatest decrease between day seven and 14. Between day 14 and 21 there was only a small decrease to 2.9μS. This indicated that there was a rapid uptake of nutrients at the start of the cycle when the plants were multiplying rapidly and by day 21 multiplication had decreased as did the uptake of nutrients (Figure 3.14). With the semi-solid system the EC was initially low (3.2μS) and at day seven it increased to 4.5 μS and thereafter dropped again to 3.5 µS which was higher than the original count. To begin with the gel in the semi-solid system appeared to be binding the nutrients, and the nutrients only became available to the plants on day seven allowing uptake. However the uptake of the nutrients is not as great when compared with the RITA® system. In RITA®, nutrients were immediately available. After 28 days the plants in RITA® system deteriorated and died due to lack of nutrients. In the semi-solid system plantlet numbers increased slowly between days 21 and 28; multiplication had slowed, but the length of the shoots increased. The pH of both media started high and then dropped after seven days. For both systems it then remained at between 3.5 to 4 (Figure 3.15).

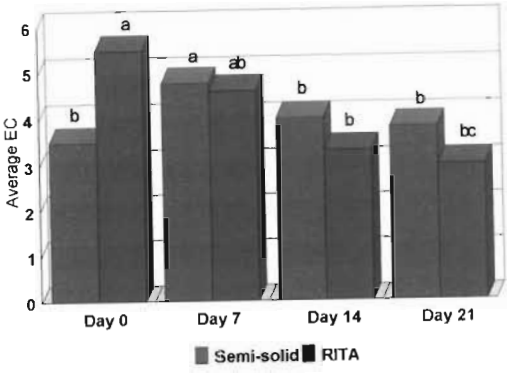


Figure 3.14. EC (μS) of medium from RITA® and semi-solid systems over 21 days ($p < 0.01$).

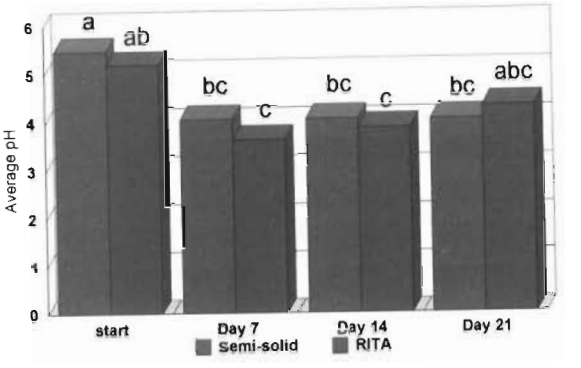


Figure 3.15. pH of medium over 21 days in the RITA® and semi-solid system ($p<0.01$)

A plant is able to take up ions selectively to some extent and accumulation of ions against a concentration gradient can occur. The uptake process requires energy and oxygen. Uptake of ions depends on environment, light and humidity interactions. Light, CO_2, temperatures and nutrient availability affect the growth rate of a plant. There are distinct differences among plant species of ion uptake (GISLERØD & SELLIAH, 2002). Optimum pH for micropropagation is between five to six. According to GISLERØD & SELLIAH (2002) the nutrients do not have to be optimal if the plants are being grown *ex vitro,* but if the plants are in a closed system the nutrients have to be accurate. Furthermore, if one changes the medium regularly it is not necessary to be so accurate with the proportions of the macro- and micro-elements. However, if the plants are left in culture for a long time on the same medium the nutrient mix has to be fairly accurate in relation to the tissue requirements. ESCALONA *et al.* (1999) stated that higher proliferation rates could be associated with pH, which might facilitate the availability of some ions. One of the advantages of temporary immersion culture on *in vitro* nutrition may be that the temporary immersion limits the movement out of the plants of ions associated with pH change. ESCALONA *et al.* (1999) found that there was a net decrease in mineral content of plants following the transfer to fresh medium during each subculture in conventional micropropagation. This was the case in some of the ions but not in others. The concentration of the nutrients in the media falls during the culture of the explants and it is assumed here, as in other studies e.g. SKIDMORE *et al.* (1988) that the nutrient depletion from the media when plants were present was due to uptake by the plants.

a. Macro-elements

The media of the semi-solid and RITA® systems have approximately the same concentration of potassium (750 mg.l $^{-1}$). At day 14 there was a significantly lower amount of K in the medium of the RITA® system and this decrease occurred on day 21 in the semi-solid system (Figure 3.16). There was no significant difference found in the amounts of K in the plants between the systems or over time (Figure 3.17).

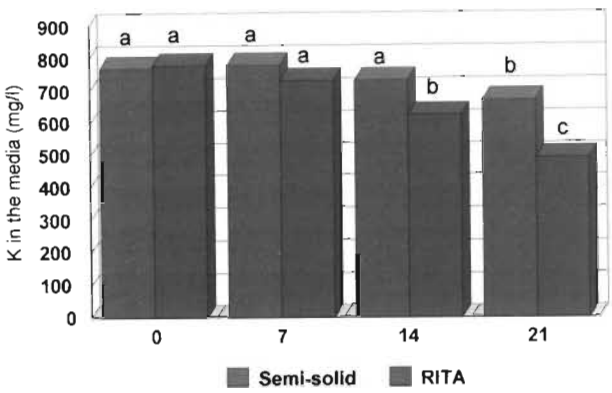


Figure 3.16. Potassium (mg.l $^{-1}$) in the M1 medium of the RITA® and semi-solid systems over 21 days ($p < 0.01$)

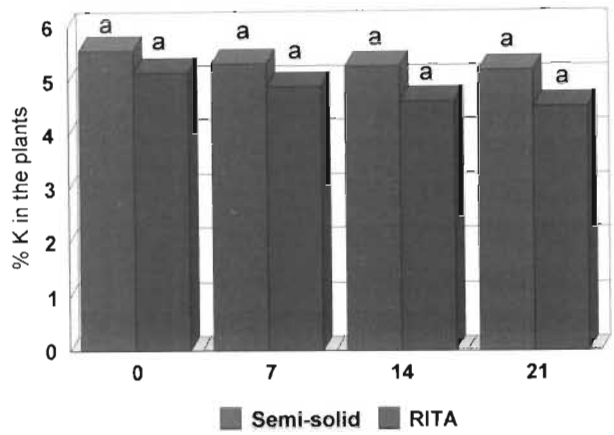


Figure 3.17. Potassium (%) found in the shoots of GU177 grown on M1 medium in the RITA® and semi-solid system over 21 days ($p < 0.01$)

As K in the plants is rarely a problem *in vitro* and the tissue levels are a reflection of supply rather than demand by the plants (WILLIAMS, 1995), it is evident that this nutrient is not a problem for either system. Uptake is increased with more availability of K but it does not have much effect on the plants (GISLERØD & SELLIAH, 2002). SEON *et al.* (2000) found with liquid culture of lilies that the K and Ca remained high in the liquid medium throughout the culture period. However with *Eucalyptus* at day 14 and again at day 21 there were significant decreases in K in the liquid media which suggests that it is one of the nutrients that could be a limiting factor, thus causing the need to transfer the shoots every 14 to 21 days. In the semi-solid media a significant decrease only occurred at day 21.

Phosphorus in the semi-solid and RITA® media was low but then on day 14 there was a significant difference in the amounts that were present in the media. By day 21 the P dropped back to a low level. It is not clear where this increased P came from at day 14 (Figure 3.18). The percentage P

found in the plants with the RITA® system was significant at 21 days. In the semi-solid system it increased but not significantly after day seven (Figure 3.19). Phosphorous is important for the energy metabolism of cells (GEORGE *et al.* 1988; DUCHEFA CATALOGUE, 1998-1999). It can be seen that these levels increased in the plants indicating that growth occurred as P is a structural element of nucleic acids.

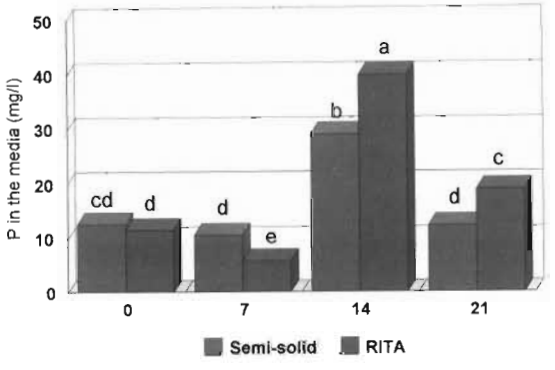


Figure 3.18. Phosphorous (mg.l^{-1}) in the M1 medium of the RITA® and semi-solid systems over 21 days ($p < 0.01$)

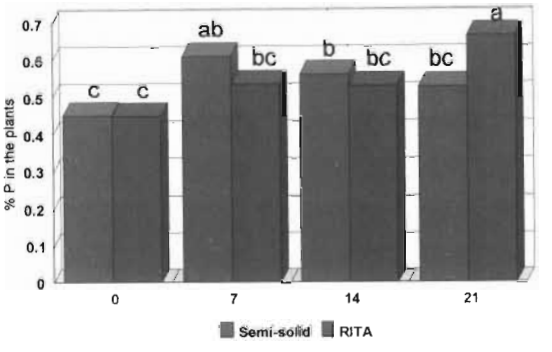


Figure 3.19. Phosphorus (%) found in the shoots of GU177 grown on M1 medium in the RITA® and semi-solid system over 21 days ($p < 0.01$)

Total nitrogen in the semi-solid and liquid systems at the start was high with a steady decrease in the medium indicating that the plants were using the nitrogen. By day 14 very low levels of N were left in the RITA® system (Figure 3.20), while in the plants the nitrogen levels increased (Figure 3.21), indicating that the plants had utilized the nitrogen for multiplication. As optimal multiplication was achieved by day 14 (Figure 3.12), this low level of N was a clear indication of plant utilization. After this the size of shoots increased. In the semi-solid system the N in the media decreased more slowly over time but by day 21 it was also considerably lower. The percentage N in the plants was significantly different at day 14 where both systems showed an increase, but this decreased again by day 21 (Figure 3.21). GISLERØD & SELLIAH (2002) stated that a relationship between N, P and K was found in closed systems and that if the P and K levels were doubled in the media the uptake of N was also doubled. SEON *et al.* (2000) found that nitrates were utilized very quickly in liquid systems. The type of N in the media influences growth and morphogenesis (GAMBORG, 1970).

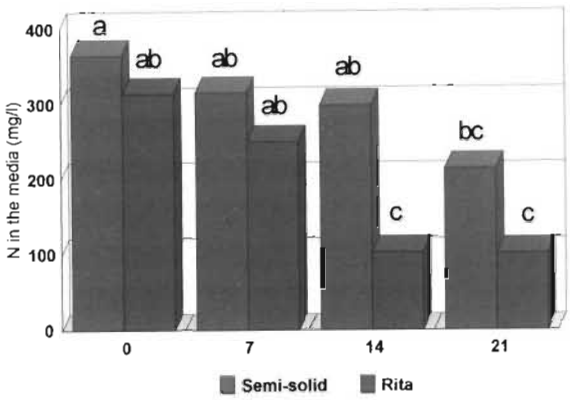


Figure 3.20. Nitrogen (mg.l $^{-1}$) in the M1 medium of the RITA® and semi-solid systems over 21 days ($p < 0.01$)

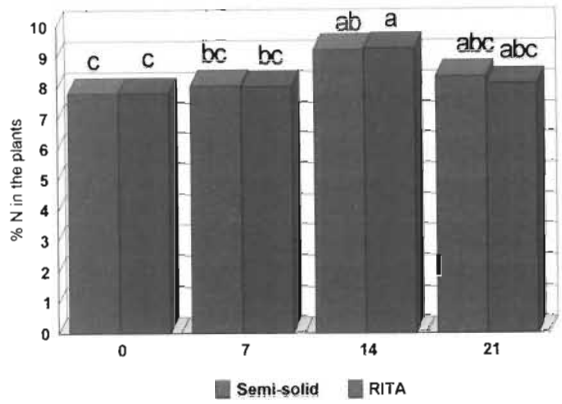


Figure 3.21. Nitrogen (%) found in the shoots of GU177 grown on M1 medium in the RITA® and semi-solid system over 21 days ($p < 0.01$)

Calcium in the semi-solid media was significantly higher than that in the liquid media. This could be due to the gelrite or to the bonds that develop within the chemicals and at day 14 there was significantly more in the semi-solid media but at day 21 it had dropped lower in both systems (Figure 3.22). Calcium in the plants produced in the semi-solid system was higher than in the RITA® system but it did decrease over time. With the liquid media there was an initial decrease of the Ca in the plants and then it remained static (Figure 3.23). GISLERØD & SELLIAH (2002) stated that a low salinity gave a better Ca uptake. Ca uptake is also linked to humidity and low humidity gives a better uptake. The EC dropped over time in both systems in the media but Ca levels in the plants did not increase. This could be due to the high humidity found in the vessels causing low Ca uptake.

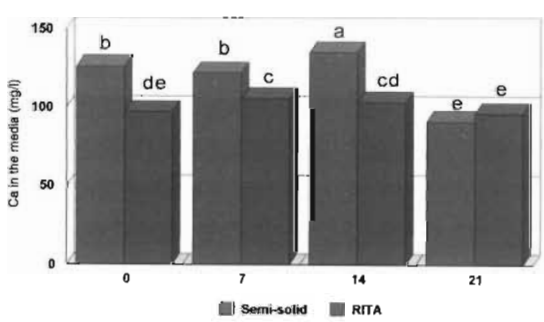


Figure 3.22. Calcium (mg.l $^{-1}$) in the M1 medium of the RITA® and semi-solid systems over 21 days ($p < 0.01$)

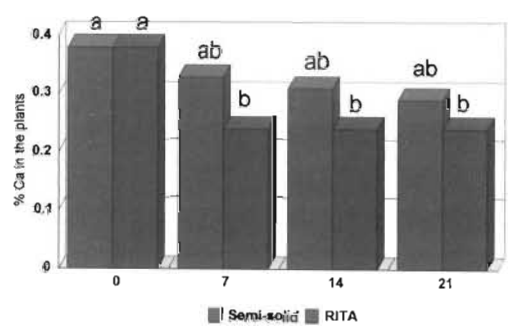


Figure 3.23. Calcium (%) found in the shoots of GU177 grown on M1 medium in the RITA® and semi-solid system over 21 days ($p < 0.01$)

On day 21 in the shoots grown in RITA® system there was a significantly lower amount of magnesium whereas in the semi-solid system it changed all the time (Figure 3.24). The percentage Mg in the plants of both systems did not significantly change over the time or between the systems (Figure 3.25). SKIDMORE *et al.* (1988) reported that on *Pinus caribaea* there were significant correlations between the uptake of Ca and Mg and Mg and P at different growth stages. However, with *Eucalyptus* there did not seem to be any correlation. SKIDMORE *et al.* (1988) stated that correlations between nutrient uptake and developmental characters would only occur in cases where nutrient availability was sub-optimal, which is generally not the case *in vitro*.

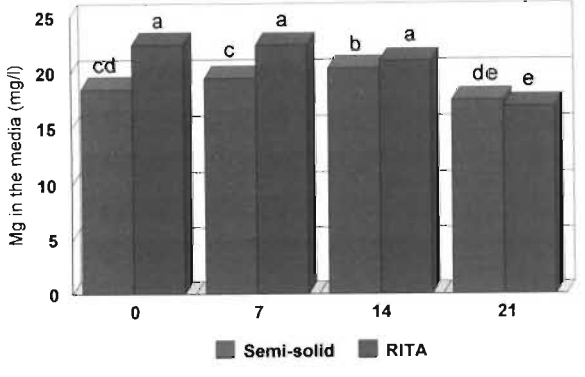


Figure 3.24. Magnesium ($mg.l^{-1}$) in the M1 medium of the RITA® and semi-solid systems over 21 days ($p< 0.01$)

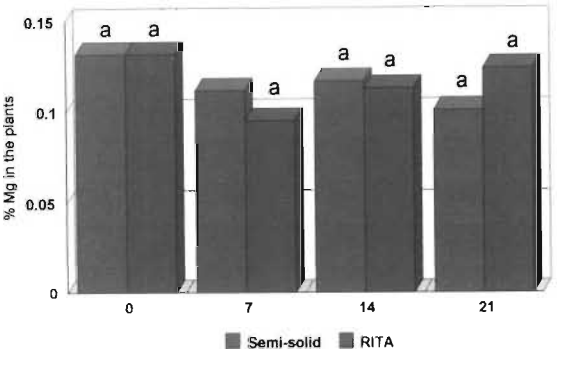


Figure 3.25. Magnesium (%) found in the shoots of GU177 grown on M1 medium in the RITA® and semi-solid system over 21 days ($p< 0.01$)

b. Micro-elements

Manganese in the liquid system started significantly higher than that of the semi-solid system and dropped over the 21 day period (Figure 3.26). In the semi-solid system this could have been affected by autoclaving of the media, combined with the gelrite present in the media as it was low initially (day 0) and then mobilized into the media (day 14) after which it decreased. In the plants the Mn was recorded as significantly higher for both systems at the start of the trial but then decreased at day seven for both systems. The Mn in the shoots in the RITA® system at day seven was significantly lower than those grown in the semi-solid system but by day 21, the reverse was observed (Figure 3.27).

Boron in the medium was high in both systems and then dropped by day seven. By day 14 boron in the liquid media rose appreciably but by day 21 it had dropped very low (Figure 3.28). In the semi-solid media boron had dropped by day seven and then rose slightly by day 14, and remained nearly constant to day 21. In the semi-solid system the boron in the plants remained between 25-35 $mg.l^{-1}$ for the entire 21 days while in the RITA® system it had risen to 60 $mg.l^{-1}$ by day 21 (Figure 3.29). Boron is a trace element and is vital to many biochmical processes (DODDS & ROBERTS, 1995). The plants in the RITA® system were taking up B in large quantities by day 21.

No significant difference occurred with the iron in the media of both systems until day 21 (Figure 3.30), the iron level decreases in the semi-solid and increases in the RITA® system. In the plants the iron drops significantly from the start to day seven in both systems (Figure 3.31). Thereafter in the plants grown on the semi-solid system dropped slowly and in the RITA® system the iron content in the plants increased to day 21 but the level in the plants did not reach the level that was found at day 0. Iron deficiencies can be caused by light as 300 lux or 12 $\mu mol.m^{-2}.s^{-1}$ with NaFe-EDTA can result in an iron deficiency in the plants (GISLERØD & SELLIAH, 2002).

Copper in the media of the semi-solid system was significantly higher at days 0 and 7 than that of the liquid system. This could be due to impurities occurring in the gel (i.e. high amounts of copper could be in the gel) (Figure 3.32). In the media of the RITA® system the Cu levels remained constantly low. The Cu levels in the plants grown in the liquid system decreased over the 21 day period while in the plants of the semi-solid system it decreased by day seven, then increased by day 14 and dropped again by day 21 (Figure 3.33).

Zinc in the media of the liquid system significantly decreased over the 21 days whereas the level of Zn in the media of the semi-solid system fluctuated and finally dropped off (Figure 3.34). In the plants Zn increased considerably over the 21 days in the RITA® system. The increase in the level of Zn in the plants was directly converse to the decrease in the media, indicating that the plants were taking up zinc in the liquid system. There was however, no significant change of zinc in the semi-solid system (Figure 3.35).

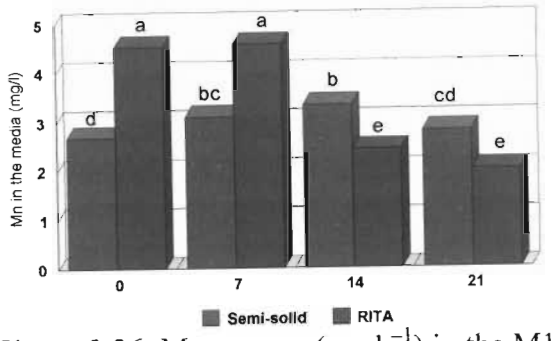


Figure 3.26. Manganese (mg.l $^{-1}$) in the M1 medium of the RITA® and semi-solid systems over 21 days ($p < 0.01$)

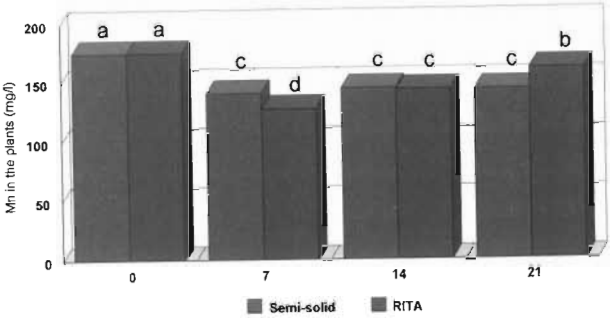


Figure 3.27. Manganese (%) found in the shoots of GU177 grown on M1 medium in the RITA® and semi-solid system over 21 days ($p < 0.01$)

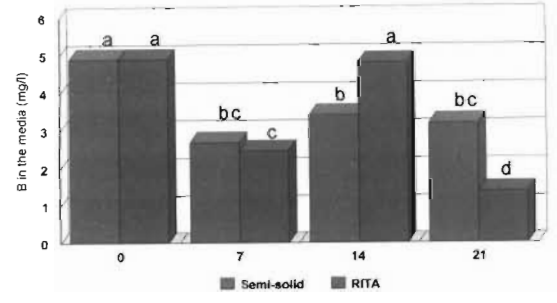


Figure 3.28. Boron (mg.l $^{-1}$) in the M1 medium of the RITA® and semi-solid systems over 21 days ($p < 0.01$)

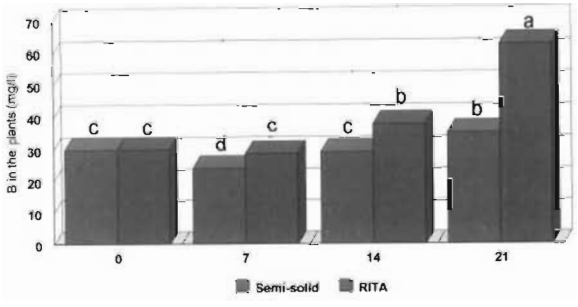


Figure 3.29. Boron (%) found in the shoots of GU177 grown on M1 medium in the RITA® and semi-solid systems over 21 days ($p < 0.01$)

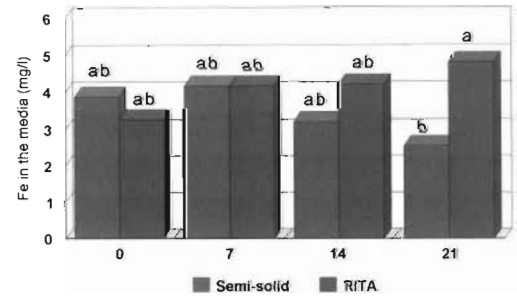


Figure 3.30. Iron (mg.l $^{-1}$) in the M1 medium of the RITA® and semi-solid systems over 21 days ($p < 0.01$)

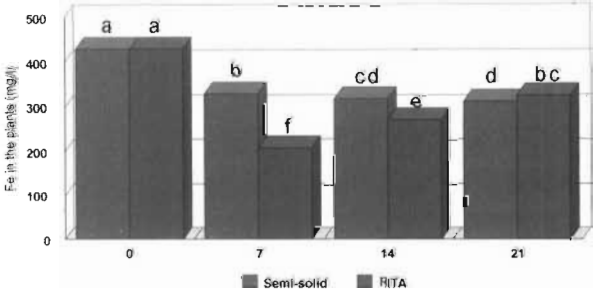


Figure 3.31. Iron (%) found in the shoots of GU177 grown on M1 medium in the RITA® and semi-solid systems over 21 days ($p < 0.01$)

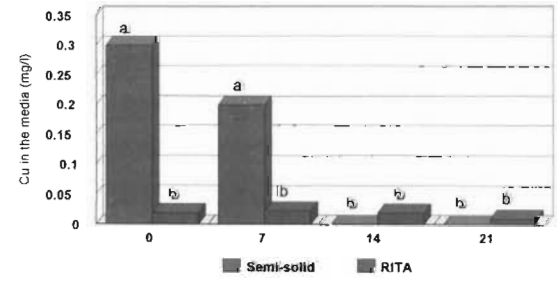


Figure 3.32. Copper (mg.l $^{-1}$) in the M1 medium of the RITA® and semi-solid systems over 21 days ($p < 0.01$)

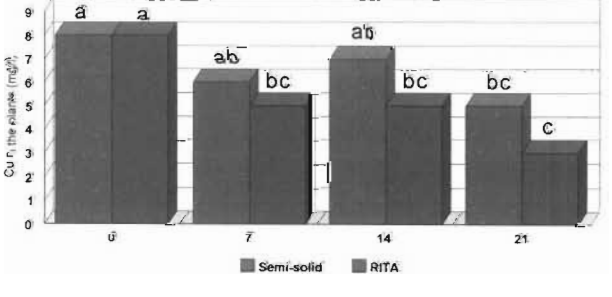


Figure 3.33. Copper (%) found in the shoots of GU177 grown on M1 medium in the RITA® and semi-solids systems over 21 days ($p < 0.01$)

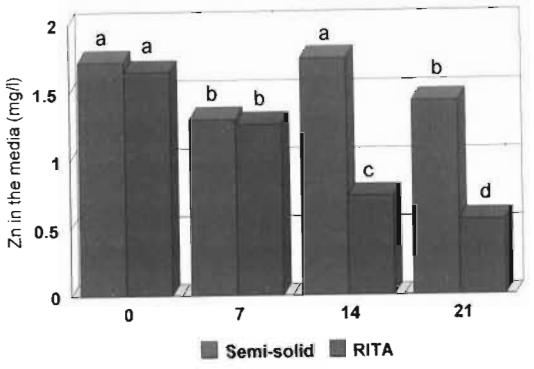


Figure 3.34. Zinc (mg.l $^{-1}$) in the M1 medium of the RITA® and semi-solid systems over 21 days ($p < 0.01$)

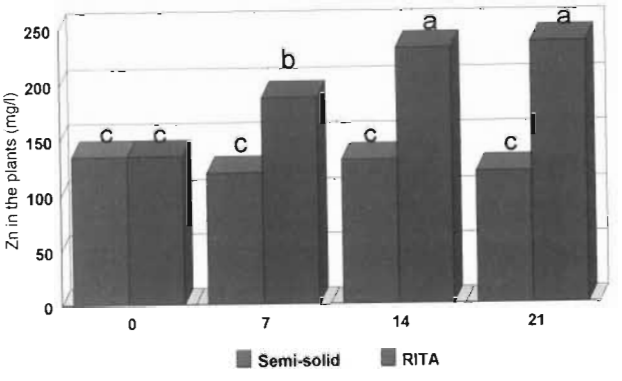


Figure 3.35. Zinc (%) found in the shoots of GU177 grown on M1 medium in the RITA® semi-solid system over 21 days ($p < 0.01$)

Changes occurred within each element at each time interval for the semi-solid and RITA® systems. The differences in uptake of the different elements in the two systems may have been due to physical environmental factors such as:

- the gel caused binding initially of some of the nutrients
- the liquid covered the plants and uptake easily occurred
- the physical factors of the vessel such as head space and size and colour of the vessel are different
- the vessel closure and forced ventilation of the RITA® system, and subsequent differences in relative humidity and gaseous composition and exchange

ALVARD *et al.* (1993) stated that the two features of the temporary immersion system which are not inherent in the classic liquid or semi-solid culture procedures are: the ability to aerate the plant tissue and also to provide contact for a programable duration between the whole of the explants and liquid medium. It is these features which could have led to more efficient uptake of the nutrients and better quality plants. HAHN & PAEK (2002) found that the number of single nodes of chrysanthemum could be maximized since the physical and chemical culture environments are controlled in optimal conditions. Air temperature, photon flux, CO_2 supply, air volume, nutrient composition, number of nodes at the initial culture are some of the factors affecting culture environments. This allowed each node to develop into a shoot in far less time.

3.4. Conclusion

The temporary immersion system (RITA®) is an efficient tool for multiplication of *Eucalyptus*. The reasons for the considerably increased multiplication may be the daily multiple air exchange which drains out gaseous compounds like ethylene, and the uptake of nutrients and hormones over the whole plant surface. The chemical environment (availability of nutrients and plant growth regulators) is important and does have considerable effect on the multiplication but it is the interrelationship of this with the physical environmental that is even more important. Physical factors such as the vessel ventilation, times of immersion and rest, size of vessel, the ability to have a physical substrate rather than a semi-solid substrate, and the physical covering of the plants with the nutrients, could definitely have contributed to increased multiplication and differences in nutrient uptake.

Maximum multiplication was achieved using 30 second flushes with 10 minute intervals. Fifty shoots per vessel was found to be the best starting number of shoots for the *Eucalyptus* clones tested. Flush and interval times influenced the multiplication, as did the media in which the shoots were placed. M1 media gave the best multiplication, although there were clonal differences in multiplication both in the liquid and the semi-solid systems. Maximum shoot multiplication in the RITA® system was achieved over 14 to 21 days, which was faster than *in vitro* propagation on the semi-solid media (28 days). There was improved multiplication in half the time, using the RITA® system. However, with many multiplication cycles on the same media the shoots became smaller, thus an elongation phase and media sequence regime was important for continued quality plants.

The semi-solid system appears to bind the nutrients to the gel and does not promote growth for the first seven days whereas in the RITA® system the nutrients for growth are available immediately. This increased multiplication in a shorter time span and the fact that the plants produced by the RITA® system are of a superior quality, are important factors to be considered in commercial plant production.

Chapter 4. Elongation, Rooting and Acclimatization

4.1. Introduction

Acclimatization refers to the gradual hardening of a shoot, plantlet or other micropropagated propagules during the transition from *in vitro* to *ex vitro* environments. Acclimatization involves a change of the physical micro-environmental conditions between the *in vitro* and *in vivo* (AITKEN-CHRISTIE *et al.* 1995). The physiological status of tissue cultured plants can have a tremendous impact on their subsequent survival under greenhouse conditions (DEBERGH *et al.* 2000). The successful *ex vitro* acclimatization of micropropagated plants determines the quality of the end product and, in commercial production, the economic viability of the enterprise (DONNELLY & TISDALL, 1993; ZIV, 1995).

As a result of the change in environment, desiccation or wilting occurs rapidly when plants are transplanted from culture to the greenhouse unless substantial precautions are taken to accommodate them. Desiccation is often the limiting factor and methods that work for one species do not necessarily ensure survival of another species. A saturated atmosphere, low light intensities, high temperatures and low rates of gas exchange between the environment and the external atmosphere, and a high concentration of carbohydrate and exogenous growth regulators in the media, characterize conventional culture environments. Plants grown in conventional micropropagation have a thin cuticular layer and poor stomatal functioning together with many other physiological inefficiencies (WETZSTEIN & SOMMER, 1983; KOZAI, 1988; DESJARDINS *et al.* 1995; DE KLERK, 2000). Major strategies for *in vitro* hardening have focused on changing the culture environment to modify these characteristics.

In addition *in vitro* plants are often smaller than those produced in the greenhouse (DONNELLY & TISDALL, 1993). It became evident in the multiplication trials of this study that the shoots, although still multiplying, became smaller in size when a continuous multiplication media was used for a long period of time. It was important, therefore, to determine if an elongation phase would assist with rooting and subsequent acclimatization. Very small plants are not practical to

place in the greenhouse as they are difficult to handle. Thus it was important to obtain strong healthy plants prior to hardening. It was felt that the media used prior to rooting and different plant growth regulators could have an effect on shoot size, rooting and hardening-off in the greenhouse. Rooting in the RITA® vessels or in the greenhouse, together with high acclimatization efficiency are all-important aspects for the production of healthy plants for commercial purposes. Some of these aspects will be summarized in this chapter to determine if plants from the RITA® system had improved rooting capabilities when compared with the plants from the conventional semi-solid system.

4.2. Materials and Methods

4.2.1. Evaluation of elongation with the use of different media

a. Effect of different media on elongation

Fifty shoots of NH58, GN107, GN108 were taken from multiplication media (Appendix 2) and placed into RITA® vessels on the following media for 14 days: M1, M2, E1, E2, MS, ½ MS, MS and ½ sucrose, ½ MS and ½ sucrose (Appendix 2). A flush time of 30 seconds and a rest time of 10 minutes was used. The effect of the media on the condition and size of the shoots was measured, after which the shoots were placed onto RM media (Appendix 2) for seven days. Average rooting percentages for the three clones in the RITA® system and in the greenhouse together with survival of the shoots after 28 days in the greenhouse, were recorded. Acclimatization for these and all further investigations were undertaken in the greenhouse under the following conditions:

- greenhouse was a poly-carbonate structure
- light of 6100 lux
- temperature was set at 25 °C and controlled by side fans
- 80 % relative humidity was achieved by fine overhead mist sprayers
- bottom heat of the beds was 30 °C

- plants were planted into 128 Unigro® trays containing a mix of palm peat, perlite and vermiculite in a ratio of 1:4:6

b. Effect of light on elongation

A cool white fluorescent light (2 500 lux), incandescent light (40W globes at 1 900 lux) and incandescent and fluorescent light (4 300 lux) were used to determine if lighting had an effect on shoot elongation. Fifty shoots each of GN108 and TAG31 were placed in vessels (six vessels per treatment) containing MS media (Appendix 2). This media had no plant growth regulators and the shoots exhibited elongation and leaf enlargement in the multiplication investigations (Chapter 3). The vessels were placed onto shelves with the different types of lights and grown with a 16 hour light/eight hour dark regime for 14 days.

4.2.2. Effect on rooting of different plant growth regulators and supports

a. Rooting plant growth regulators in the RITA® system

Fifty shoots of three different clones (TAG31, GN107, and GN108) were used on the standard MS media (Appendix 2) in the RITA® system for 14 days. After which various concentrations of IAA and IBA (0, 1, 2, 3 $mg.l^{-1}$) were added to this MS media. IBA at the different concentrations was added to the media prior to the media being autoclaved. Using a 0.22μ filter the various concentrations of IAA were filter sterilized into the media after the media had been autoclaved to prevent degradation of the plant growth regulator. Shoots were left on the different media for 14 days. Thereafter rooting percentages and the morphological effects that the different concentrations of plant growth regulators had on the shoots in the RITA® vessels were recorded at 14 days.

b. Effect on rooting of change of media at different cycles in the RITA® system

The type of media in which the shoots are grown prior to placement onto rooting media can affect rooting. Different combinations of multiplication, elongation and rooting media for different time periods were applied to GN108 shoots to assess what effect, if any, the media sequences and

time periods had on rooting (Table 4.1). Initial number of shoots per vessel was 50 and a flush time of 30 seconds every 10 minutes was used. After 14 or 21 days on M1 media (Appendix 2) shoots were measured and divided into two groups (size 0 to 3 or 3 to 7 cm). These were then placed onto different media – either rooting or elongation media for different time periods and then moved onto a further cycle of rooting or elongation media (Table 4.1).

Table 4.1. Media (multiplication, elongation and rooting) treatments and number of days the shoots were placed on the medium (Appendix 2)

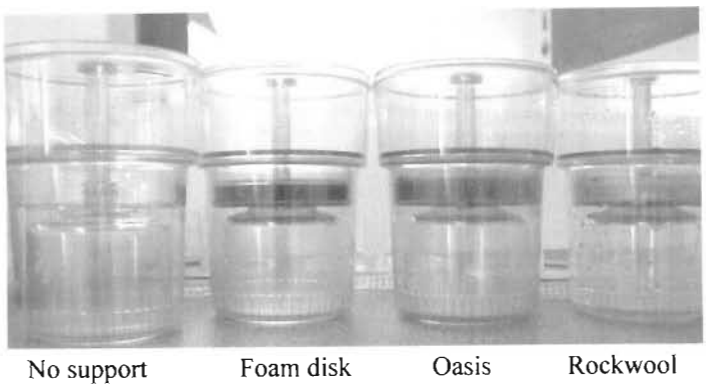
Treatment	Type of media and number of days on each media at the different cycles		
	Cycle 1	Cycle 2	Cycle 3
1	M1, 14 days	RM, 7 days	MS, 14 days
2	M1, 14 days	RM, 14 days	MS, 14 days
3	M1, 14 days	RM, 21 days	MS, 14 days
4	M1, 14 days	RM, 28 days	MS, 14 days
5	M1, 14 days	MS, 7 days	RM, 14 days
6	M1, 14 days	MS, 14 days	RM, 14 days
7	M1, 14 days	MS, 21 days	RM, 14 days
8	M1, 14 days	MS, 28 days	RM, 14 days
9	M1, 21 days		RM, 14 days
10	M1, 21 days		E2, 14 days
11	M1, 14 days	MS, 7 days	E2, 14 days

Rooting in the vessels was measured after the completion of the different cycles of treatments (Table 4.1). The shoots with and without roots were then planted in the greenhouse for a period of 28 days for acclimatization. Rooting that occurred in the greenhouse and survival of shoots were recorded for all the different treatments after 28 days in the greenhouse.

c. Effect on rooting of different supports

The RITA® vessel has a standard polyurethane foam disk for support of the shoots. Once the plants produce roots, these grow into the foam and it is difficult to remove the plants without damage to the roots. Thus it became important to find better support methods. Oasis, Rockwool,

and vessels with no foam disk were used as supports to determine if they were more effective than the foam disks (Figure 4.1). The morphological effect on the shoots and roots was recorded.



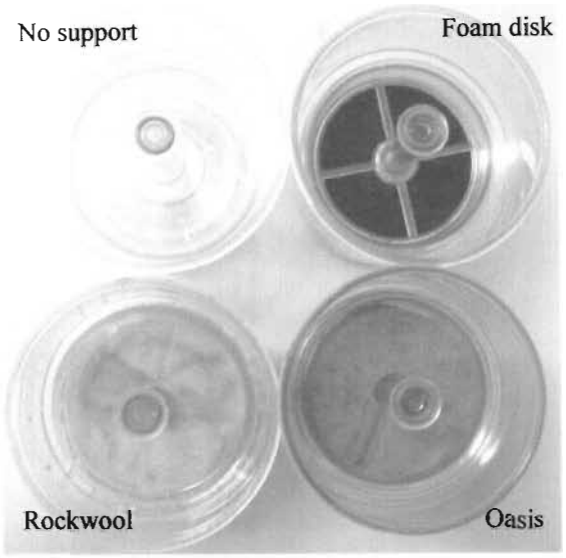


Figure 4.1. Different support systems for the plants at the rooting stage

4.2.3. Comparisons of rooting in the semi-solid *vs.* the RITA® system

Shoots of four sub-tropical clones (GU175, GU177, GU178, GU180) and two cold-tolerant clones (GN108, NH58) from the respective multiplication media (semi-solid and RITA®) were placed onto RM media (Appendix 2) in the two systems. After seven days in the rooting media, plants (with and without roots) were placed in the greenhouse. Rooting and survival of acclimatized plants from both systems was recorded and a comparison was undertaken. The phenotypic differences were compared.

4.2.4. Data analysis

All data was analyzed statistically using multiple analysis of variance (ANOVA) and differences were compared using Duncan's multiple range test.

4.3. Results and Discussion

4.3.1. Elongation of shoots

a. Media used for elongation and its effect on rooting thereafter

Although high multiplication rates were achieved over many multiplication cycles in the RITA® system, the shoots that were produced tended to become smaller in size, which caused a problem when placing onto rooting media as the plants were too small to handle with each subsequent subculture. For most herbaceous species rooting is not a problem provided the propagation and the elongation stages are appropriate. However the problems are more pronounced in woody species and it is important to elongate the plants prior to rooting (DEBERGH *et al.* 2000).

Fifty shoots of three different clones (NH58, GN107 and GN108) were placed onto the different media and their elongation was measured. It was found that the shoots in M1 and M2 media (Appendix 2) did not increase in size (one to 1.5 centimeters was the starting size), which was to be expected, as these media are for multiplication. However on MS and ½ MS media good elongation occurred with the plants reaching four to six centimeters and the shoots were dark green and healthy (Figure 4.2, Table 4.2).

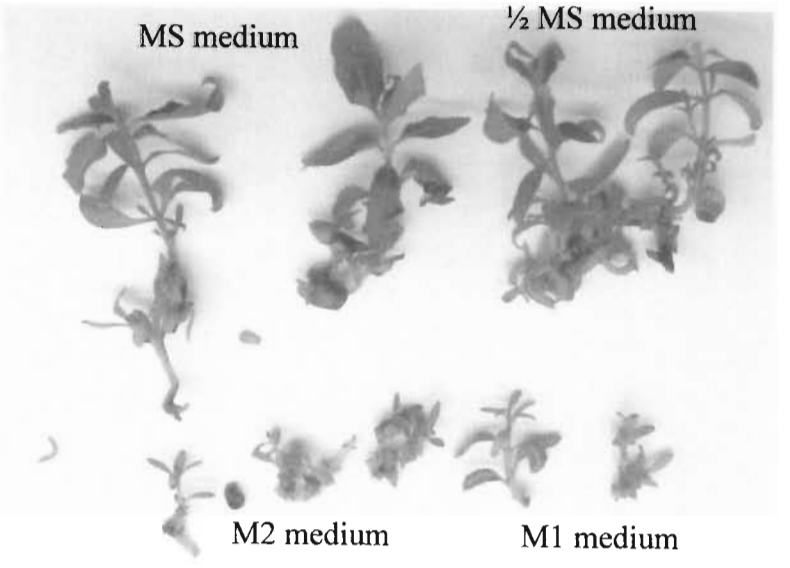


Figure 4.2. Shoots grown on MS, ½ MS, M1 and M2 media

Table 4.2. The effect of the different media (Appendix 2) on the shoot quality and the size of the shoots of three different clones

+++ – Large dark green, healthy shoot ++ – Healthy shoot of medium length
\+ – Small shoot, healthy --- – Poor quality shoot, pale green in colour

Media	GN107		GN108		NH58	
	Size (cm)	Shoot quality	Size (cm)	Shoot quality	Size (cm)	Shoot quality
M1	1.5	+	1	+	1	+
M2	1.5	+	1	+	1.5	+
MS	6	+++	6	+++	5	+++
½ MS	5	+++	4	+++	5	+++
MS ½ Sucrose	3	++	4	+	4	++
½ MS ½ Sucrose	4	+	3	---	4	---
E1	5	++	4	---	5	++
E2	4	+	3	+	5	---

For all the clones where full MS and ½ MS were used large healthy looking plants were produced. MS with ½ sucrose produced smaller but healthy plants. ½ MS and ½ sucrose gave plants between three and four centimeters but the shoots of GN108 and NH58 were pale green with small pale leaves. E1 and E2 produced fairly big shoots for all the clones ranging from three to five centimeters, but GN108 on E1 and NH58 on E2 resulted in poor quality shoots although they had elongated.

After the measurements were recorded the shoots from all clones were then placed onto rooting media (Appendix 2) in the RITA® system for seven days and the rooting was recorded. The elongation media affected the rooting in the vessels. M1 and ½ MS gave significantly higher average rooting percentages for all three clones (Figure 4.3). The other media had no effect on the number of shoots that rooted on the rooting media. This indicated that M1 and ½ MS should be used prior to placement onto rooting media to obtain optimal rooting in the RITA® system. The results from this trial indicate that ½ MS medium on the clones tested was a favourable medium to use for elongation, producing good quality plants and thereafter giving good rooting and acclimatization results in the greenhouse.

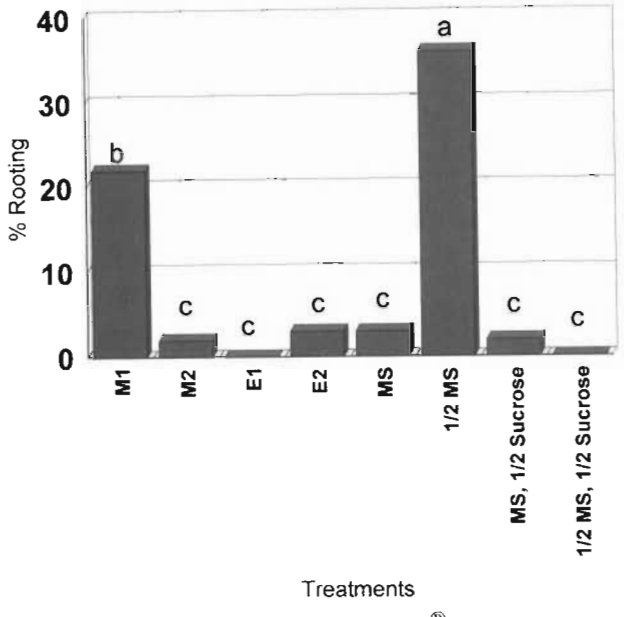


Figure 4.3. Average rooting of three clones in the RITA® vessels

After 28 days in the greenhouse the rooting which occurred after placement was recorded as well as the survival numbers (shoots that had no roots but remained green and healthy). Three clones were used for this trial, two cold-tolerant clones as rooting is normally poor in these clones. Rooting (30-35 %) in the greenhouse was achieved on M1 and M2 (Figure 4.4). As these two media were used in multiplication it gives an indication that for good rooting the plants should be in a healthy multiplying and growing state. The plants on the other media were in a less viable state for rooting when placed into the greenhouse. Where half concentration of nutrients with high sucrose (E2 and ½ MS) were used prior to rooting the percentages were 22 and 27 % respectively. Plants grown in MS, MS with ½ sucrose, ½ MS and ½ sucrose and E1 gave very low rooting percentages after 28 days (between 5 to 17 %). These media were not effective prior to rooting as they affected the final rooting percentages. Plants from M1 had the highest survival of the unrooted shoots after 28 days and plants from the two media with half the concentration of sucrose had the lowest survival rate (Figure 4.5). High sucrose in the media prior to placement into the greenhouse could have served as a carbon source. This could have been stored in the plant and resulted in longer survival in the greenhouse. This could also have been the case with the shoots in M1 media which had the highest sucrose concentration (2.5 $g.l^{-1}$). The shoots still

had the potential to root as they remained green and healthy (Figure 4.4). This is supported by the reports of DAMIANO *et al.* (1987) who found that sucrose between two and six percent in the media favoured root development in *Eucalyptus,* and WILLIAMS (1995) who found that a change in the sucrose levels affected the morphological development of the shoots.

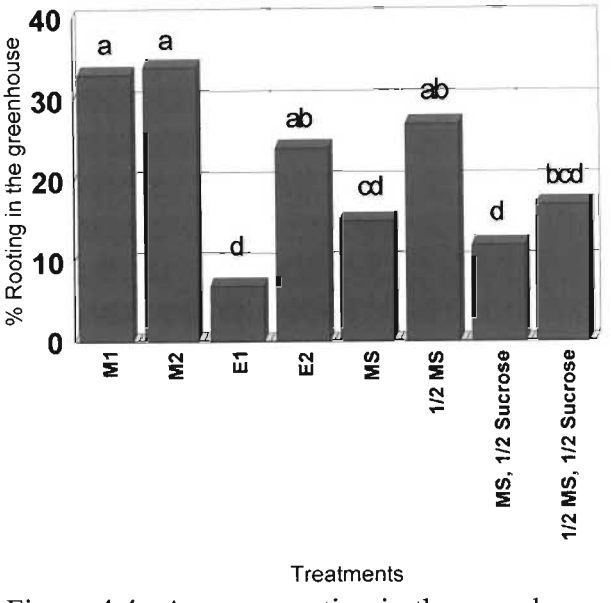


Figure 4.4. Average rooting in the greenhouse from the different pre-rooting media for three cold-tolerant clones

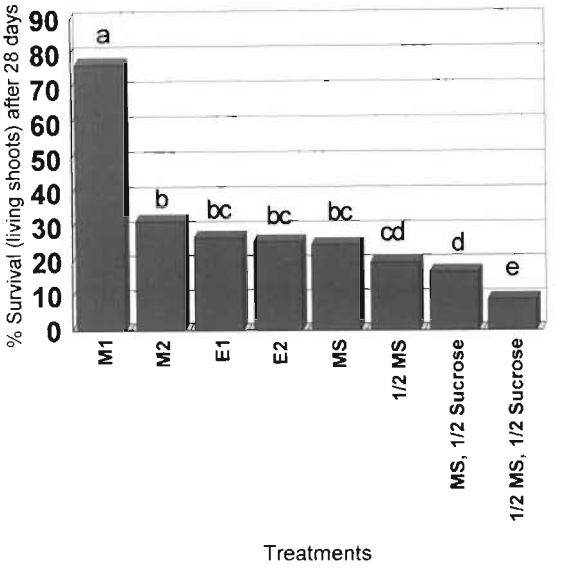


Figure 4.5. Average survival of the of the shoots in the greenhouse after 28 days for the three cold-tolerant clones grown on different pre-rooting media

The composition of media used prior to the rooting media had an effect on the rooting and survival of shoots in the greenhouse. Although rooting was low for some of the treatments in the RITA® vessels once placed in the greenhouse the rooting percentage increased. ZIV (1995) stated that reducing the levels of some mineral nutrients, especially in scale-up liquid cultures, could contribute to improved morphogenetic responses (e.g. elongation) that provide for more efficient acclimatization. The elongation of the plant is thus important to produce good quality plants for acclimatization. Elongation of plants needs to be undertaken periodically throughout the multiplication cycles to maintain a better quality product.

b. Effect of light on elongation

Light has a major influence on growth, development and morphogenesis of the plants (ELLIS & WEBB, 1993). Photosynthesis and photomorphogenesis are radiation dependant (ZIV, 1995). For photosynthesis to occur 400-700 nm at high irradiance is important while for photomorphogenesis blue, red and far red light are required (ZIV, 1995). It was found that different lighting affected the growth of plants in the RITA® vessels.

Incandescent light, which has the red and far-red wavelengths, as the only light source or incandescent light used in conjunction with fluorescent light, caused heating of the growth room and condensation in the vessels. This condensation in the vessels (Figure 4.6) may have been the cause of callusing of some of the plants (Figures 4.6 and Table 4.3). The plants grew well but were very etiolated with little or no lignification and unable to support themselves once transferred (Figure 4.7). The plants were pale in colour and the shoot size varied. Fluorescent light only resulted in a wide range of shoot sizes, however the plants were a dark green in colour and were healthy and could be transferred to rooting medium easily.

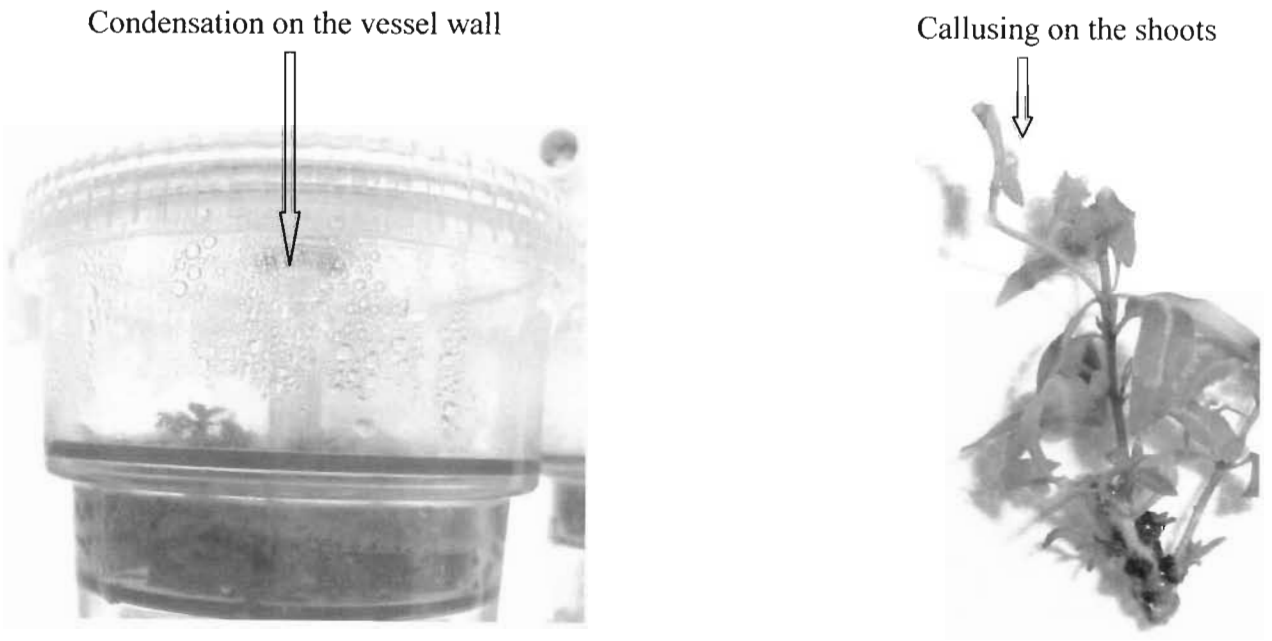


Figure 4.6. Condensation on the vessel walls and lid (left) which caused callusing of the plants (right)

Table 4.3. Effect of different light sources on the shoot size and quality of the plants

	Shoot quality	Size of shoot (cm)
Incandescent	Variation in shoot size was large. Shoots became long and were pale. Poor quality plants to transfer to rooting media. Callusing occurred as condensation was high in the vessels	2 – 9
Fluorescent	Wide range of shoot size with plants darker green and sturdy. Good quality plants produced which could be transferred to rooting media.	0.5 – 7
Incandescent & Fluorescent	Shoots grew very well but became etiolated and were pale green with no lignification. Shoots could not support themselves once transferred. Poor quality plants produced.	5 – 8

Figure 4.7. Etiolated plants with pale small leaves produced under incandescent and fluorescent light (4 300 lux)

ZIV (1995) stated that when vertical radiation is used, a radiation gradient forms and causes etiolation. This was possibly the case in this study, as the plants with incandescent and fluorescent or incandescent light grew much faster and filled the vessels more quickly. The plants tended to grow towards the light causing the etiolation. The cool white fluorescent light gave a wide range of sizes of plants from 0.5 to seven centimeters (the average starting size of the shoots was one to two centimeters) and the plants were of an improved quality. This could have been due to the fact that the plants were not multiplying as fast as with the other light regimes and less radiation gradient occurred. It is apparent that the optimal lighting for the *Eucalyptus* plants in the RITA® vessels is cool white fluorescent light (2 500 lux).

4.3.2. Rooting in vessels and support mechanisms

a. Effect of plant growth regulators on rooting

Different auxins at various concentrations affect the development of roots. It is important to determine the correct concentrations and plant growth regulators to use for optimal normal rooting, and for these reasons different concentrations of IBA and IAA were tested. There was a statistically significant difference between the clones on the various treatments (indicated in red letters on Table 4.4). There was also a statistical difference between the averages of the various clones at each treatment (indicated in blue letters on Table 4.4). Furthermore, significant differences were found within each clone with the different plant growth regulators and plant growth regulator concentrations (indicated in black letters on Table 4.4). Abnormal rooting occurred when the concentrations of plant growth regulators were too high (indicated in pink).

On all three concentrations of IBA and IAA clone TAG31 had high rooting. However at the two higher concentrations of each plant growth regulator abnormal rooting occurred and roots developed all over the stems and leaves of the shoots indicating that the plant growth regulator concentrations were too high (Table 4.4). Where no plant growth regulators were present very little rooting occurred. With the two GN clones rooting was not high on 3 $mg.l^{-1}$ IBA and no rooting occurred where no plant growth regulators were present. However, IAA at 3 $mg.l^{-1}$ gave abnormal rooting over the entire surface of the shoots of both cold-tolerant clones, indicating that this concentration was too high. IBA at 1 $mg.l^{-1}$ gave the highest percentage of normal rooting out the base of the stem only (47 to 53 %) for the cold-tolerant clones and the rooting percentage for TAG31 was 98 % with good roots. At 2 $mg.l^{-1}$ IBA and IAA both gave lower rooting (22 to 34 %). As the plant growth regulator concentrations increased there was a decrease of normal rooting percentages i.e. an increase of abnormal roots occurred. IAA gave lower normal rooting for all three *Eucalyptus* clones at the three concentrations compared with that of IBA. The best normal root formation for all the clones tested was obtained with the use of 1 $mg.l^{-1}$ IBA.

DE KLERK (2000) reported that for optimal rooting of apple IAA was better than IBA or NAA, which indicates that each species has a preference for a particular plant growth regulator.

ABDULLAH *et al.* (1989) found that auxins were crucial for root initiation of *Pinus brutia* and that the response varied according to the concentrations of auxin applied. This was the case for *Eucalyptus.* However on the whole, IAA tended to be less effective for rooting *in vitro* *Eucalyptus* shoots for the sub-tropical clone tested as abnormal rooting occurred. Good rooting resulted on the two cold-tolerant clones with the use of IAA at 1 $mg.l^{-1}$ and 2 $mg.l^{-1}$, however this was not as effective as 1$mg.l^{-1}$ IBA. As stated elsewhere in previous chapters DENISON & KIETZKA (1993) found cold-tolerant clones to be poor rooters. The 53 % for GN108 and 47 % for GN107 obtained with 1 $mg.l^{-1}$ IBA was therefore an improved rooting percentage.

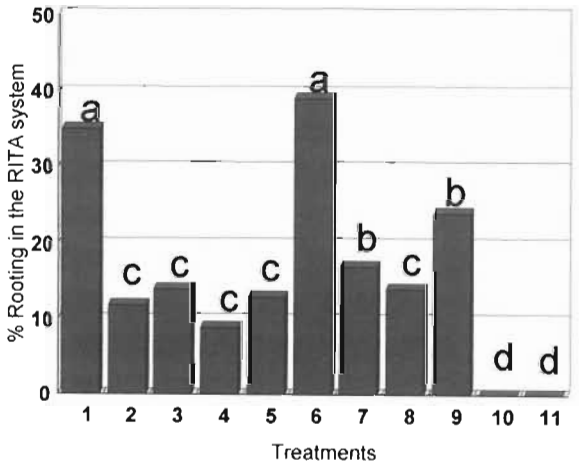
Table 4.4. Rooting percentages (% R) and effects of IBA and IAA at different concentrations on three clones in the RITA® vessels ($p<0.01$).

Clone		TAG 31 a		GN107 b		GN108 c	
Hormone		% R	Effect	% R	Effect	% R	Effect
IBA ($mg.l^{-1}$)	0 e	5 c	Spontaneous rooting. Shoots healthy and strong	0 e	Shoots elongate and big	0 e	No rooting. Shoots healthy and big
	1 b	98 a	Plants rooted easily and healthy and big	47 b	Shoots healthy and big with roots directly from shoots	53 b	Shoots healthy and big with roots directly from shoots
	2 d	100 a	Roots produced over the leaves and stems. Abnormal rooting	34 cd	Shoots healthy and big with roots directly from shoots	22 d	Shoots healthy and big with roots directly from shoots
	3 d	100 a	Roots developed over the leaves and stems, more prolifically than in 2 $mg.l^{-1}$. Rooting hormone too high	5 e	Shoots a little callused and not healthy	0 e	Shoots died off
IAA ($mg.l^{-1}$)	0 e	5 c	Spontaneous rooting. Shoots healthy and strong Abnormal rooting	0 e	Shoots elongated well	0 e	Shoots elongated well
	1 c	90 b	Roots developed well. Shoots healthy Abnormal rooting	46 bc	Shoots healthy and big with roots directly from shoots	33 c	Shoots healthy and big with roots directly from shoots
	2 cd	100 a	Rooting out of leaves Abnormal rooting	33 d	Shoots healthy and big with roots directly from shoots	29 cd	Shoots healthy and big with roots directly from shoots
	3 a	100 a	Rooting out of leaves. Abnormal rooting	100 a	Rooting out of leaves. Abnormal rooting	100 a	Rooting out of leaves. Abnormal rooting

b. Effect of change of media on rooting at different cycles

It was found with the elongation trials that the media used prior to the rooting media caused different rooting percentages. Thus it was important to investigate the effects of the various media at all cycles and what effect different sized shoots had on rooting percentages (Table 4.1. shows the different cycles and treatments to which the shoots were subjected to). The shoots were measured and the rooting percentages that occurred for the different treatments in the RITA® system were recorded. Thereafter the shoots were divided into two groups (0 to 3 cm and 3 to 7 cm) and the average rooting percentages were recorded after 28 days in the greenhouse.

Treatments 1 and 6 gave significantly higher rooting in the RITA® system, with treatments 9 and 7 giving the next highest. The remaining treatments gave low rooting percentages. In treatments 10 and 11 no rooting occurred in the RITA® system (Figure 4.8).



Key:
1. M1, 14days > RM, 7days > MS, 14days
2. M1, 14days > RM, 14days > MS, 14days
3. M1, 14days > RM, 21days > MS, 14days
4. M1, 14days > RM, 28days > MS, 14days
5. M1, 14days > MS, 7days > RM, 14days
6. M1, 14days > MS, 14days > RM, 14days
7. M1, 14days > MS, 21days > RM, 14days
8. M1, 14days > MS, 28days > RM, 14days
9. M1, 21days > RM, 14days
10. M1, 21days > E2, 14days
11. M1, 14days > MS, 7days > E2,14days

Figure 4.8. Average rooting in the RITA® system with the various treatments (Treatments 1-11, seen in Table 4.1) ($p<0.01$)

After 28 days in the greenhouse there was a significant difference between the size and rooting potential across all the treatments, with the larger shoots generally having a better potential to root. Treatments 4,5,7,8,9,10 and 11 gave better rooting percentages from the bigger shoots. However treatments 1,2,3 and 6 gave better rooting from the smaller shoots (Figure 4.9). There was a significant difference in rooting percentages between the two shoot sizes with treatment 6

(M1 14 days, MS 14 days and rooting media 14 days). Treatment 6 showed 55 % rooting with the small shoots. Treatment 9 (M1 21 days and rooting media for 14 days) gave 42 % rooting with the large shoots.

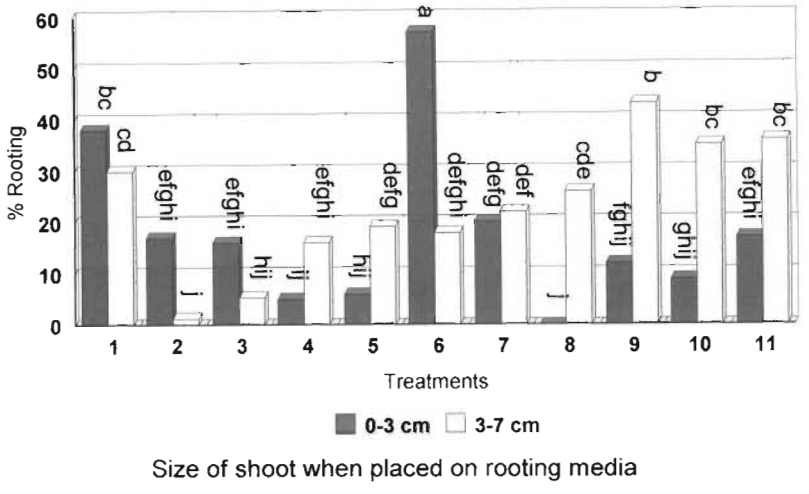


Figure 4.9. Average rooting of different sized shoots grown on different media sequences for different time periods (treatments 1-11 can be seen in Table 4.1.) ($p<0.01$)

With the combined plant sizes, treatment 1 (M1 14 days, rooting medium, 7 days and MS 14 days (Table 4.1)) resulted in the highest rooting (Figure 4.9). This treatment involved a short period on rooting medium, transferred to an elongation medium. The seven days on rooting medium appeared to play an important role as those shoots placed on the rooting medium for 14, 21 and 28 days prior to MS medium had low rooting percentages (treatments 2,3 and 4).

The plants on M1 medium for 14 days then onto MS medium for differing numbers of days, followed by rooting medium for 14 days resulted in different rooting percentages. Treatments 5, 7 and 8 gave low rooting percentages. The duration of time the shoots remained on MS was significant as indicated by treatment 6 (MS for 14 days). This gave the increased rooting percentages whereas plants subjected to treatments 5, 7 and 8 were on MS medium for 7, 21 and 28 days respectively and gave low rooting percentages.

Plants on multiplication medium (M1) followed by rooting medium (treatment 9) gave 43 % rooting with the bigger shoots. Treatment 10 and 11 where the shoots were taken from multiplication media to an elongation medium also had improved rooting with the bigger sized plants (35 and 36 % respectively) compared with the small sized plants.

ABDULLAH *et al.* (1989) tested the effects of auxin and cytokinins on the induction of roots of *Pinus brutia.* They found that both auxin and cytokinin and the interactions between them, affected the quantity and quality of induced roots. Their results drew attention to the fact that care needs to be taken in the choice and application of the medium for the initial induction of roots. VAN TELGEN *et al.* (1992) used *Calathea ornate* and *Malus* to determine the effect of propagation and rooting conditions on acclimatization. They also found that acclimatization was influenced, not only by the conditions during the rooting and acclimatization phases, but also by the conditions during the propagation stage. PINKER (2000) also found that the stem properties and rooting performance were affected considerably by the duration of the last subculture on multiplication medium. This was certainly the case with *Eucalyptus,* as not only did the media composition affect the acclimatization and rooting but the time that the plants remained on the medium also had a distinct effect. MS medium for 14 days and RM medium for 14 days, and RM for seven days and MS medium for 14 days were the two sequences of media which achieved the highest number of acclimatized plants. The number of days on each medium was important, as the other periods did not achieve the same results.

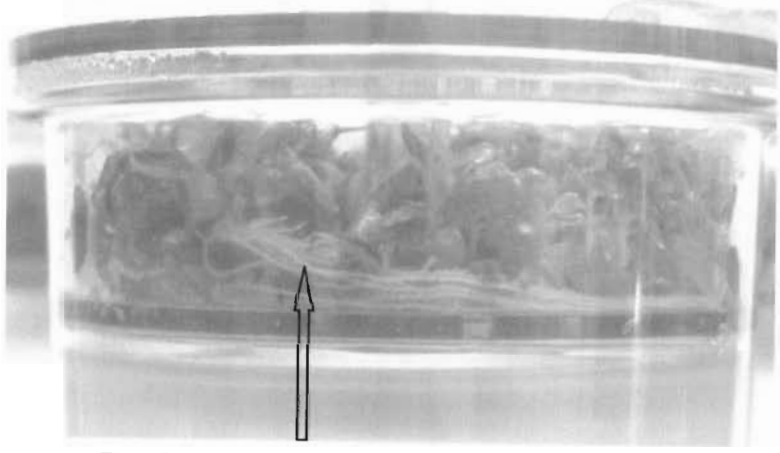
c. Supports for rooting plants

As the plants in the RITA® vessels produced roots these grew into the sponge supports, presenting a problem for removal of the plants. It was often very difficult to remove the rooted shoots from the vessels when the roots had grown though the sponge as the plants then had to be cut out to prevent breakage of the roots. This was very time consuming and plants were easily damaged. It was apparent that another method of support for these shoots was needed. Different supports were tested and results were recorded (Table 4.5). The best method was found to be no support other than the plastic chamber on which the plants were placed. No damage occurred to the roots when transferring the plants to the greenhouse. The plants were merely picked up and

placed in the media (Figure 4.10). With the foam disks a great deal of damage was done to the roots on transfer to the greenhouse. Oasis was a good alternative to the foam as the plants were easily removed with little or no damage to the roots. However the plants did rot off around the base when left too long on this support as it retained media. With Rockwool the plants rotted very quickly, therefore it was best to have no support at all.

Table 4.5. Response of the plants using different support for rooting shoots

Support	Response
Sponge	Roots grew into the sponge and were damaged on transplanting to the greenhouse.
Oasis	Shoots produced roots and grew healthily. However if left for more than 10-14 days the shoots started to rot as the oasis held too much moisture. The plants were readily removed from Oasis without damage.
Rockwool	Shoots rotted very quickly as the Rockwool held too much moisture even when the flushes were reduced to a single flush per day.
No support, plastic chamber	Shoots rooted readily and could be removed very easily, no rotting occurred



Rooting which occurred in the vessels with no support (the plastic chamber only) left

Figure 4.10. Vessels with no foam support (left). Roots formed in the vessels

4.3.3. Comparisons of rooting in semi-solid *vs.* the RITA® system

This study indicated that plant quality is important when rooting and it can be seen that the plants produced by the RITA® system were superior to those of the semi-solid system, which prompted trials to improve rooting and acclimatization. Minimal callus was evident on the leaves, bases and stems of plants in the RITA® system, with roots developing directly from the base of the stems. This was not the case with the semi-solid system as the plants often formed callus at the base of the stems from which roots grew. This caused problems at the acclimatization stage (Figure 4.11). The levels of O_2 in the vessels affect the root system and where an anaerobic or low O_2 condition occurs rooting is reduced or abnormal roots form (JACKSON, 2002). With the semi-solid system a lower concentration of O_2 in the gel may have resulted in poor root development, whereas with the RITA® system there was a continuous supply of O_2 which may have improved rooting.

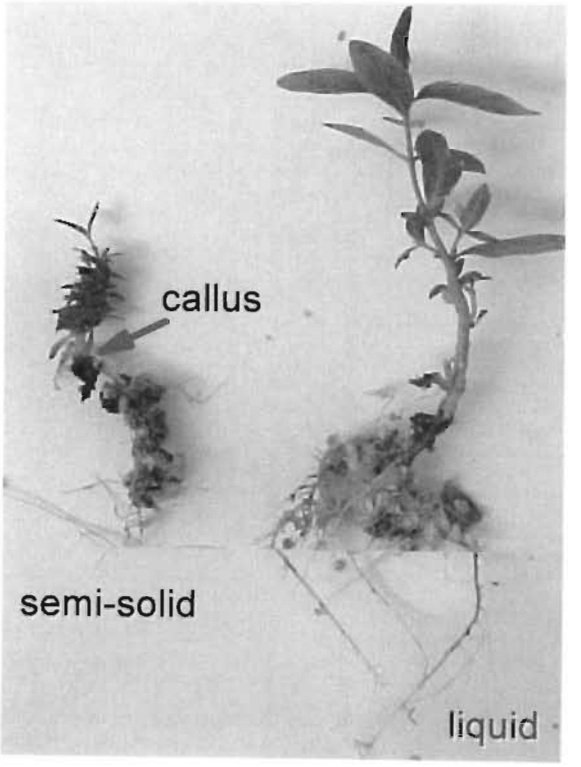


Figure 4.11. Root development from plants grown on the two different systems after four days in the greenhouse

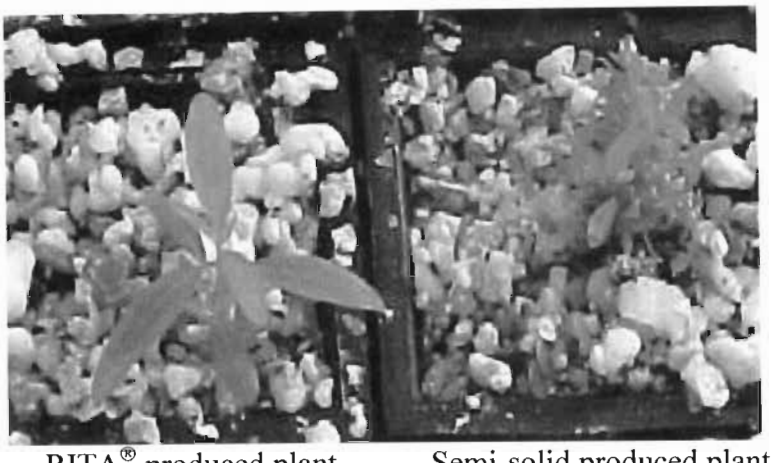
Plantlets in the RITA® vessel rooted readily *in vitro* using modified MS medium containing IBA. Roots also developed *ex vitro*. Nevertheless, clones were found to have different acclimatization potentials (Table 4.6). In this regard, percent rooting was determined for four sub-tropical clones known to be 'easy rooters' (GU175, GU177, GU178 and GU180), and for two 'difficult-to-root' cold-tolerant clones (GN108 and NH58). The sub-tropical clones showed no difference in percentage rooting between the semi-solid and the RITA® rooting environments. In contrast, rooting of the cold-tolerant clones was 6.5 % and 53 % in semi-solid and RITA® systems respectively. It seems, therefore, that one of the greatest values of the RITA® system is to facilitate the rooting steps in recalcitrant clones.

Table 4.6. Acclimatization success of plants sent to the greenhouse with and without roots from the RITA® and the semi-solid systems (expressed as % of total plants transferred from laboratory to greenhouse)

	Semi-solid (% rooting)		**RITA® (% rooting)**	
Clone	**With roots**	**Without roots**	**With roots**	**Without roots**
GU175	43	30	32	9
GU177	47	23	52	33
GU178	50	29	53	15
GU180	39	28	36	18
Average rooting percent for the sub-tropical clones	36		35	
GN108	20	1	63	37
NH58	5	0	67	43
Average rooting percent for the cold-tolerant clones	6.5		53	

The shoots produced by the two systems were different in quality. The RITA® system produced larger, darker green broader leafed plants whereas those on the semi-solid system developed into small, pale plants with small leaves (Figure 4.12). In the semi-solid system the vessels are closed and the type and tightness of the vessel closure determines the concentrations of CO_2, O_2 and C_2H_4 and water vapour in the culture gaseous atmosphere (ZIV, 1995). ZIV (2002) stated that CO_2 enrichment is shown to increase photosynthesis. With the RITA® system there is a continuous air exchange which could have led to improved photosynthetic ability thus giving

better quality plants. This gaseous exchange also allows better development of the stomata and cuticular layer thus allowing plants from the RITA® system to acclimatize more easily.



RITA® produced plant Semi-solid produced plant

Figure 4.12. Differences in the appearance of the shoots from the two different systems, after greenhouse acclimatization

With *Eucalyptus,* acclimatization was improved in the plants that came from the RITA® system as the plants produced were of a better quality. The air exchange that occurred in the RITA® vessels could have led to better stomatal and outer epidermal layer development which may have given the plants an improved chance of survival. The improved acclimatization results obtained in this study were similar to those found by BERTHOULY & ETIENNE (2002) in that plant material propagated by temporary immersion performed better during the acclimatization phase than material obtained on semi-solid or liquid media. AFREEN, ZOYBAYED & KOZAI (2002) did a comparison of the RITA® system and a specially designed temporary immersion root zone bioreactor system (TRI-bioreactor) when inducing rooting with *Coffea sp.* They found that the TRI-bioreactor gave better acclimatization (84 %) compared with the RITA® system (20 %). The stomata were functioning normally and photosynthesis was higher in the TIR-bioreactor compared with the RITA® system. With the RITA® system 53 % acclimatization of *Eucalyptus* plants was achieved.

4.4. Conclusion

Attempts were made to increase the size of the plants prior to rooting as it was hypothesized that bigger shoots may improve rooting and subsequent acclimatization and survival of the plants. MS and ½ MS resulted in the best elongation. However the type of elongation media used affected rooting in the vessels. The medium used prior to the rooting medium was found to be important as it had an effect on the rooting and survival of shoots. Fluorescent light resulted in the best type of plants *in vitro* with the RITA® system. Although incandescent light facilitated good elongation, the plants were not robust and were etiolated. For rooting in the RITA® system, the plant growth regulator type and concentration that resulted in the highest rooting was IBA at 1 $mg.l^{-1}$ for two cold-tolerant and one sub-tropical *Eucalyptus* clones. This plant growth regulator at that concentration gave the highest rooting (47 to 53 %) for the cold-tolerant clones and 98 % for the sub-tropical clone. As was found with the elongation trials the medium used prior to the rooting media contributed to different rooting percentages. The period of time, to which the plants were subjected to a particular medium, played a role in rooting, as did the size of the plants (those with larger shoots produced better rooting).

Plants placed into the vessels with no support facilitated ease of removal from the vessels without damage to the roots. The sub-tropical clones showed no percentage difference in rooting between the semi-solid and the RITA® system rooting environments. However the cold-tolerant clones were substantially different, with it being advantageous to use the RITA® system to produce cold-tolerant plants. The plants produced in the RITA® system were of a superior quality and acclimatized more readily than those grown on the semi-solid system. For a future study to optimize rooting in liquid culture and to determine if the acclimatization percentages could be improved, it would be of great value to build a system similar to that of AFREEN *et al.* (2002) in which there is forced CO_2.

Chapter 5. Cost Benefit Analysis of the RITA® System Compared with the Semi-solid System

5.1. Introduction

Within Mondi's *Eucalyptus* tree improvement strategy, the increased focus on specific clones and production targets prompted investigation of ways to reduce costs and increase yields in a shorter time. The previous chapters have described how the RITA® system was identified as a potentially important method of increasing multiplication yields and rooting of *Eucalyptus* clones. As seen, the productivity of the liquid temporary immersion system exceeded that of the semi-solid system. In addition, plants produced from the liquid temporary immersion system were of a higher quality than the plants from the semi-solid system. ETIENNE *et al.* (1997); BORROTO & ETIENNE (1998); TEISSON & ALVARD (1998); BERTHOULY & ETIENNE (2002); KOKKO *et al.* (2002); KOSKY *et al.* (2002) and SAGE & SHROEDER (2002) used the RITA® system for various different species. They reported a decrease in costs and an increase in the number and quality of plants produced. The value of this system is discussed in this chapter in terms of yields, costs and application to the *Eucalyptus* plantation industry in Mondi Forests and in South Africa.

5.2. Yields

Increased multiplication in a shorter period was achieved in the temporary immersion system RITA® compared with the semi-solid system (Figure 5.1).

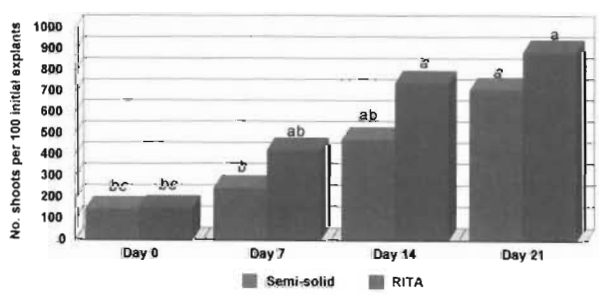


Figure 5.1. Average multiplication for three sub-tropical clones and two cold-tolerant clones on the semi-solid and RITA® systems

This increased multiplication in the RITA® system was achieved in a smaller production space compared with that of the semi-solid system (Figure 5.2), approximately 1 792 and 3 200 plants per m^2 were produced at the onset of multiplication, for the semi-solid and RITA® systems respectively.

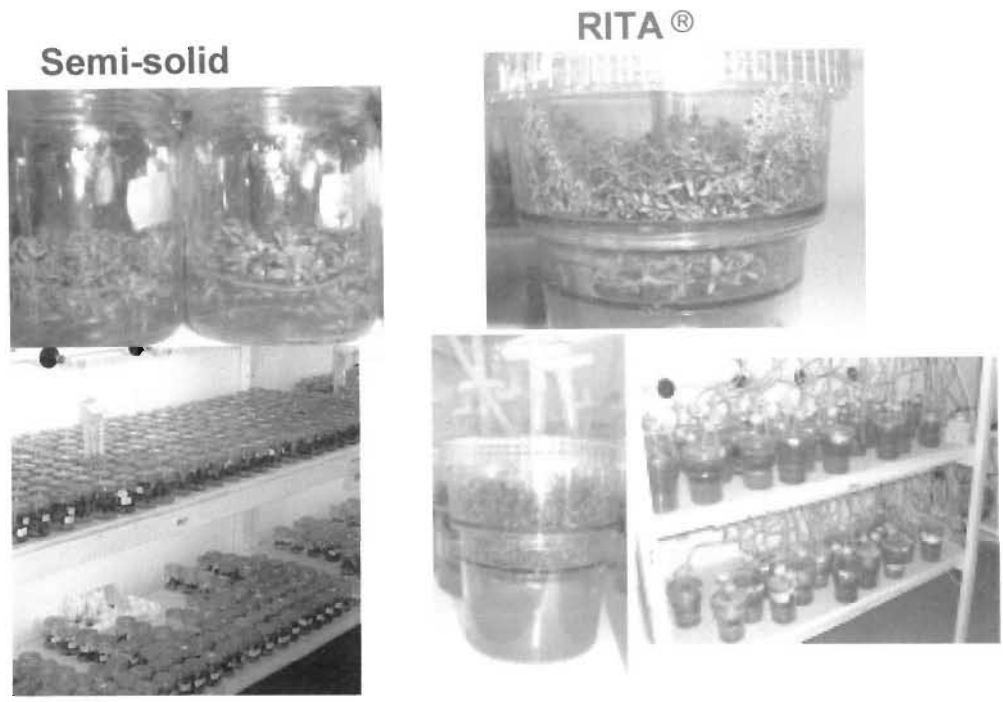


Figure 5.2. Multiplication in the jars of the semi-solid system and RITA® system and the space required for the respective systems

In addition to the multiplication rates that were achieved in a smaller space with the RITA® system, the final acclimatized yields (i.e. after greenhouse establishment and ready for planting out) were the most important in terms of evaluating the success of the method. The final yields (plants that were produced and ready for planting in the commercial hedges) from four sub-tropical clones (GU175, GU177, GU178, GU180) and two cold-tolerant clones (GN108, NH58 - average to poor rooters) from Table 4.6 in Chapter 4, were calculated (Table 5.1).

Table 5.1. Final yield (% of plants produced ready for planting/the % planted into the greenhouse for acclimatization) produced from the averages of four sub-tropical clones and two cold-tolerant clones for the semi-solid system and the RITA® system

Clone type	Semi-solid	RITA®
Sub-tropical clones	36 %	31 %
Cold-tolerant clones	6.5 %	53 %

The yields for the sub-tropical clones from the two systems are very similar, whereas the yields from the cold-tolerant clones are vastly different. This increased production of the cold-tolerant clones has the potential to contribute to the forestry industry. In Mondi Forests, cold-tolerant clones are much sought after, as they have increased yields in the field and superior pulp properties, hence they are extremely important (DENISON, 1999). A problem with cold-tolerant clones it that they have very low rootability, but with the use of the RITA® system rootability and productivity of these clones can be increased (53 %).

5.3. Costs

A cost analysis was done (Table 5.2) using the average yields for all the clones (Table 5.1). Calculations are based on data obtained to date which indicate that with 100 initial explants for both systems, 10 000 plants can be obtained with the RITA® system in three months, while in the semi-solid system it took six months to achieve that number. The cost analysis has the main expenses for tissue culture included (Table 5.2).

Table 5.2. Costs to produce 10 000 plants (from 100 starting plants) in the semi-solid and RITA® system. Data based on average rooting percentage (cold- tolerant and sub-tropical clones). Costs in South African Rand

Materials or activity	**Semi-solid (6 months)**	**RITA (3 months)**
Media	6 772	1 133
Transfer	5 700	1 710
Media preparation (labour)	5 700	1 140
Autoclaving	232	81
Washing	5 700	282
TOTAL	**24 104**	**4 346**
Initial outlay on vessels	7 143	65 000
FINAL TOTAL	**31 247**	**69 346**

Using the RITA® system the costs of the disposable items and running expenses are far lower than that of the semi-solid system. The costs of media and media preparation are reduced substantially by the elimination of a gelling agent in the liquid media and the dispensing time of the media. With the semi-solid media each aliquot of 25 ml has to be dispensed into each jar. The reduction in autoclaving is due to lower quantities of vessels and media to be autoclaved at each transfer. With the RITA® system the transfer time is considerably shortened as the shoots can be cut and 50 shoots are dropped into the vessel. However, with the semi-solid system each jar must be opened and seven shoots per jar are placed with care so that each stem is at a good depth in the semi-solid medium. If new nutrients are required during a cycle the middle unit of the RITA® vessel may be lifted out and placed into a clean vessel with new nutrients (Figure 5.3). In contrast in the semi-solid system, each individual shoot has to be handled. Using the RITA® system fewer vessels are used and therefore the washing costs are reduced. Less space was required for the production of plants in the RITA® vessels compared to those in the semi-solid system (Figure 5.2).

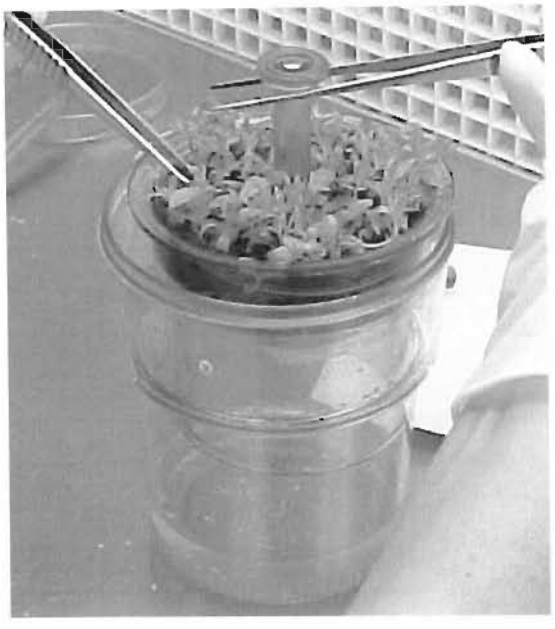


Figure 5.3. Transfer of the inner compartment of the RITA® vessel to new medium (no handling of individual shoots)

The initial outlay for the RITA® vessels is high but in addition to the vessels being re-used this is soon offset by the multiplication rates and turn-over of the plants produced (Table 5.2). The average yields (cold-tolerant together with sub-tropical) obtained from the RITA® system are higher than those in the semi-solid system. The costs involved in producing plants in the temporary immersion system are lower as more plants are produced in a shorter time from the medium. The medium also costs less, as there is no solidifying agent. Further, for a commercial laboratory, the RITA® system offers flexibility in that newly approved commercial clones can readily replace the commercial clone being produced. The RITA® system is more efficient in producing higher numbers of difficult to root clones than the semi-solid system.

The reduction in costs parallels the findings of other researchers of vast cost savings using different plants. ETIENNE (2000) found that the use of the temporary immersion system combined with direct sowing of somatic embryos of coffee eliminated a labour intensive stage in tissue culture. They found that the production time was reduced by three months and the handling time was reduced by 6.3 % compared with the standard micropropagation system. The shelving requirements were also reduced by 13 %. ETIENNE (2000) states that it is reasonable to expect major economic

gains since labour and shelving represent 70 % and 10 % respectively of micropropagation costs. LORENZO *et al.* (1998) calculated a cost reduction of 46 % for sugarcane propagation in a temporary immersion system compared with that on the agar medium. While ESCALONA *et al.* (1999) saved 20 % of production costs per pineapple plant at multiplication stage in a temporary immersion system in comparison with conventional cultures. With *Phalaenopsis* culture PREIL & HEMPFLING (2002) are expecting a considerable reduction of costs especially in manual labour using the temporary immersion.

5.4. Advantages and disadvantages of the RITA® and semi-solid systems in *Eucalyptus* micropropagation

In *Eucalyptus* micropropagation there are advantages and disadvantages of the semi-solid and the RITA® systems and at this stage these two systems should be utilized in conjunction to produce the quality and quantity of *Eucalyptus* plants needed for production purposes. This observation has been confirmed by research on other species where the use of both systems is recommended (solid-liquid-solid) to obtain optimal advantages from both systems (PREIL, 1991; GRIGORIADOU *et al.* 2002; PAMFIL, 2002). For the production of *Eucalyptus* the advantages and disadvantages are shown in Table 5.3, with many of the former outweighing the latter thus enabling the RITA® system to become more cost effective and produce higher yields especially for the more difficult cold tolerant clones.

Table 5.3. Advantages and disadvantages of the semi-solid and RITA® system in *Eucalyptus* micropropagation

	Semi-solid	**RITA®**
Contamination	Loss of low numbers per container	Loss of high numbers per container
Starting material	Shoots placed into semi-solid system	Needs semi-solid phase to obtain sterile plants in the vessels
Plant size	Small	Large
Hardening off	Poor	Good
Senescence if not given new media on time	Slow	Quick
Speed of transfer	Slow	Quick
Media transfer	28-32 days	14-21 days
Callusing	Callusing occurs	Minimal
Solidifying agent	Need agar or Gelrite	None needed
Hyperhydricity	Occasionally occurs	Occasionally occurs
Labour for dispensing media	1 person about 15 min /litre	Minimal
Wear and tear and time in the autoclave	High running cost	Low running cost
Space on shelves in the growth room	Larger space needed 1 792 plants per m^2 at onset of multiplication	Smaller area needed 3 200 plants per m^2 at onset of multiplication
Pump for air	No cost	Cost in pump
Labour for transfers	High labour	Low in labour (transfer easily)
Direct costs (South African Rand) as at (October 2002)	24 104	4 346
Outlay of vessels	7 143	65 000
Time to grow 10 000 plants	6 months	3 months
Flexibility in receiving system	Inflexible	Flexible
Efficiency in "difficult clones"	Inefficient	Increasingly efficient
Potential for somatic embryos	Medium	High

5.5. Application of the RITA® system to the *Eucalyptus* plantation industry in South Africa

The applications of the RITA® system to the *Eucalyptus* industry in South Africa are as follows:

- Clones with good field performance and higher pulp yields can be selected even if they are difficult clones to root (cold-tolerant)
- Improved rooting and higher quality plants produced
- There is a reduction in multiplication times
    - This is a direct benefit to the breeding and clonal *Eucalyptus* programs in South Africa
    - This leads to quicker deployment of commercialized clones
- There is a decrease in costs for producing difficult clones

5.6. Conclusion

The costs involved in producing plants in the temporary immersion system are lower than the semi-solid system, as more plants are produced in a shorter time. Although the initial outlay for the liquid system is high, it is offset by the reduced labour and media costs of the RITA® system, together with significantly higher rooting and survival percentages. For the production of "difficult rooters" (cold-tolerant clones) the RITA® system facilitates higher production numbers thus enhancing the value of the system in the production of these clones. The system has great potential for *in vitro* production of *Eucalyptus* plants although the semi-solid system has to be utilized in conjunction with the RITA® system to obtain contaminant free cultures in the RITA® vessels.

6. Concluding Remarks and Future Research

6.1. Concluding remarks

The results indicate that for *in vitro* culture of *Eucalyptus*, particularly cold-tolerant clones, the RITA® system provides benefits as yet not obtained with the more commonly used semi-solid protocols for axillary bud propagation. However, with *Eucalyptus*, an initial short-term semi-solid stage is recommended as a quick and economical means of establishing microbe-free plants. In the RITA® system, multiplication increases with the use of the correct number of starting shoots in the vessels, as well as the appropriate exposure to media at suitable intervals. Plant quality (hardiness and size) for clones tested to date are superior with the RITA® produced plants than the quality of the plants grown in the semi-solid media. In addition, cold-tolerant *Eucalyptus* clones (e.g. GN108 and NH058) which have proved extremely difficult to multiply, root, and subsequently acclimatize using semi-solid protocols, have been shown to respond very favourably to the RITA® environment. Costs per 10 000 plants produced using the RITA® system are less than those for the semi-solid system. The RITA® system thus has great potential for *in vitro* production of *Eucalyptus* plants commercially, provided that contaminant-free explants can be obtained via a semi-solid system.

6.2. Future research

Various aspects of *in vitro* propagation invite further investigation and research and these are:

- Optimize control of contamination
- Find a contamination screening technique to enable the placement of shoots directly from the field into the RITA® system which would reduce costs and time
- Use of larger RITA® vessels for increased production
- Automatic media change (continuous nutrient replenishment)
- Carbohydrate source studies
- More extensive elemental utilization studies

- Improvement on acclimatization conditions – AFREEN *et al.* (2002) are currently making use of large containers with CO_2 bubbled at the base which give good quality plants and many shoots can be placed in the system at a time
    - Bridge between the RITA® system and greenhouse
    - *In vitro* root primordia initiation
    - Root cooling
- Use of the RITA® vessels for the production of synchronous somatic embryos of *Eucalyptus* plants as many other researchers have found the RITA® system to be advantageous in embryo production.

If these and other issues are addressed, *in vitro* propagation will become very cost effective and will be placed within reach of smaller institutions and commercial concerns.

REFERENCES

Abdullah, A.A., Grace, J. & Yeoman, M.M. 1989. Rooting and establishment of *Calabrian* pine plantlets propagated *in vitro:* influence of growth substances, rooting medium and origin of explants. *New Phytology.* **113**:193-202

Afreen, F. Zobayed, S.M.A. & Kozai, T. 2002. Photoautotrophic culture of *Coffea arabusta* somatic embryos: Development of a bioreactor for large-scale plantlet conversion from cotyledonary embryos. *Annals of Botany.* **90**:21-29

Ahuja, M.R. 1993. Micropropagation á la carte. In: M.R. Ahuja (ed). Micropropagation of Woody Plants. Kluwer Academic Publishers. Netherlands. pp 3-9

Aitken-Christie, J. & Davies, H.E. 1988. Development of a semi-automated micropropagation system. *Acta Horticulturae.* **230**:81-87

Aitken-Christie, J., Kozai, T. & Takayama, S. 1995. Automation in plant tissue culture. General introduction and overview. In: J. Aitken-Christie, T. Kozai & M.A.L. Smith (eds). Automation and Environmental Control in Plant Tissue Culture. Kluwer Academic Publishers, Netherlands. pp 1-18

Akula, A., Becker, D. & Bateson, M. 2000. High-yielding repetitive somatic embryogenesis and plant recovery in a selected tea clone, 'TRI-2025', by temporary immersion. *Plant Cell Reports.* **19:**1140-1145

Albany, N., Vilchez, J., Jiménez, E., García, L., de Feria, M., Pérez, N., Sarría, Z., Pérez, B. & Clavelo. 2002. Use of growth retardants for banana (*Musa* AAA cv. Grand Naine) shoot multiplication in temporary immersion systems. . First international symposium for *in vitro* mass propagation of plants. Cost 843 working group. Norway. pp 108

Alvard, D., Cote, F. & Teisson, C. 1993. Comparison of methods of liquid medium culture for banana micropropagation. Effects of temporary immersion of explants. *Plant Cell, Tissue and Organ Culture.* **32**:55-60

Amiri, M.E. 2001. Mineral uptake by banana (*Musa acuminate* L.) expants *in vitro*. *Acta Horticulturae.* **560**:378-385

Azim, A., Noin, M., Landré, P., Prouteau, M., Boudet, A.M. & Chriqui, D. 1997. High frequency plant regeneration from *Eucalyptus globulus* Labill. hypocotyls: Ontogenesis and ploidy level of the regenerants. *Plant Cell, Tissue and Organ Culture.* **51**:9-16

Bach, A., Malik, M., Ptak, A. & Kedra, M. 2000. Light effects on ornamental micro-plant shoots and bulbs quality. *Acta Horticulturae.* **530**:173-179

Barbas, E., Jay-Allemand, C., Doumas, P., Chaillou, S. & Cornu, D. 1993. Effects of gelling agents on growth, mineral composition and naphthoquinone content of *in vitro* explants of hybrid walnut tree (*Juglans regia* x *Juglans nigra*). *Annual of Science and Forestry* **50**:177-186

Baurens, F.C. 1998. Personal communication. baurens@cirad.fr.

Bayley, A.D. & Blakeway, F. 2002. Deployment strategies to maximize value recovery from tree improvement: The experience of two South African companies. *South African Forestry Journal.* **195**:11-22

Bell, D.T., Van der Moezel, P.G., Bennett, I.J., McComb, J.A., Wilkins, C.F., Marshall, S.C.B. & Morgan, A.L. 1993. Comparisons of growth of *Eucalyptus camaldulensis* from seeds and tissue culture: Root, shoot and leaf morphology of 9-month-old plants grown in deep sand and sand over clay. *Forest Ecology and Management.* **57:**125-139

Bergmann, B.A. & Stomp, A.M. 1992. Influence of taxonomic relatedness and medium composition on meristematic nodule and adventitious shoot formation in nine pine species. *Canadian Journal of Forest Research.* **22**:750-755

Berthouly, M. & Etienne, H. 2002. Temporary immersion system: A new concept for use of liquid medium in mass propagation. First international symposium for *in vitro* mass propagation of plants. Cost 843 working group. Norway. pp 37-38

Beruto, M., Curir, P. & Debergh, P. 1999. Influence of agar on *in vitro* cultures: II. Biological performance of *Ranunculus* on media solidified with three different agar brands. *In Vitro Cellular and Developmental Biology - Plant.* **35:**94-101

Bonga, J.M., 1977. Applications of tissue culture in forestry. In: J. Reinert & Y.P.S. Bajaj (eds) Applied and Fundamental Aspect of Plant Cell, Tissue and Organ Culture. Springer-Verlag. Berlin. pp 93-105

Bornman, C.H. & Vogelmann, T.C. 1984. Effect of rigidity of gel medium on benzyladenine-induced adventitious bud formation and vitrification *in vitro* in *Picea abies. Physiologia Plantarum.* **61**:505-512

Borroto, C.G. (22/8/1997). Internet news group – discussion (plant-tc@tc.umn.edu or cborroto@ceniai.inf.cu.)

Borroto, C.G., & Etienne, H. 1998. Temporary-Immersion Bioreactor System Reduces Micropropagation Costs. *Agricell Report.* **30(1)**:2

Bugbee, B. 1996. Nutrient management in recirculating hydroponic culture. Crop Physiology Laboratroy, Utah State University. pp 1-5

Cassells, A.C. 1991. Problems in tissue culture: Culture contamination. In: P.C. Debergh & R.H. Zimmerman (eds). Micropropagation. Technology and Application. Kluwer Academic Publishers. Dordrecht. pp 31-44

Cassells, A.C. 1997. Pathogen and microbial contamination management in micropropagation – an overview. In: A.C. Cassells (ed). Pathogen and Microbial Contamination Management in Micropropagation. Kluwer Academic Publishers. Netherlands. pp 1-13

Cassells, A.C. 2000. Aseptic micro hydroponics: A strategy to advance micro plant development and improve micro plant physiology. *Acta Horticulturae.* **530**:187-194

Cassells, A.C. & Collins, I.M. 2000. Characterization and comparison of agars and other gelling agents for plant tissue culture use. *Acta Horticulturae.* **530**:203-212

Cheng, B., Peterson, C.M. & Mitchell, R.J. 1992. The role of sucrose, auxin and explant source on *in vitro* rooting of seedling explants of *Eucalyptus sideroxylon. Plant Science.* **87**:207-214

Chu, I. 1995. Economic analysis of automated micropropagation. In: J. Aitken-Christie, T. Kozai & M.A.L. Smith (eds). Automation and Environmental Control in Plant Tissue Culture. Kluwer Academic Publishers, Netherlands. pp 19-28

Cirad. (1/29/1999). RITA® Temporary Immersion System for Plant Tissue Culture. http://www.cirad.fr/produits/rita/en/fonction.htm

Cirad homepage (5/2/2002) RITA® Temporary Immersion System for Plant Tissue Culture. http://www.cirad.fr/produits/rita/en/internet.htm

Cooke, D.C., Waites, W.M. & Leifert. C. 1992. Effects of *Agrobacterium tumefacients, Erwinia carotovora, Pseudomonas syringae* and *Xanthomonas compestris* on plant tissue cultures of *Aster, Cheiranthus, Delphinium, Iris* and *Rosa*: disease development *in vitro* as a result of latent infections *in vitro. Journal of Plant Disease Protection.* **99**:469-481

Cooper, A. 1996. The ABC of NFT. Casper Publications. Narrabeen, NSW. pp 1-11

Cornu, D. & Michel, M.F. 1987. Bacterial contaminants in shoot cultures of *Prunus avium* L. choice and phytotoxicity of antibiotics. *Acta Horticulturae.* **212:**83-86

Cresswell, R.J. & De Fossard, R.A. 1974. Organ culture of *Eucalyptus grandis. Australian Forestry.* **37**:55-69

Damiano, C., Curir, P. & Cosmi, T. 1987. Short note on the effects of sugar on the growth of *Eucalyptus gunnii in vitro*. *Acta Horticulturae*. **212**:553-556

Damiano, C., Caboni, E., Frattarelli, A., Giorgioni, M., Liberali, M., Lauri, P. & D'Angeli, S. 2000. Innovative micropropagation of temperate fruit trees: the case of pear. *Acta Horticulturae*. **530**:181-185

Damiano, C., La Starza, S.R., Monticelli, S., Gentile, A., Caboni, E. & Frattarelli, A. 2002. Propagation of *Prunus* and *Malus* by temporary immersion. First international symposium on liquid systems for *in vitro* mass propagation of plants. Cost 843 working group. Norway. Poster

Das, T. & Mitra, G.C. 1990. Micropropagation of *Eucalyptus tereticornis* Smith. *Plant Cell, Tissue and Organ Culture*. **22**:95-103

Davies, W.J. & Santamaria, J.M. 2000. Physiological markers for micro plant shoot and root quality. *Acta Horticulturae*. **530**:363-375

Debergh, P.C. 1983. Effects of agar brand and concentration on the tissue culture medium. *Physiologia Plantarum*. **59**:270-276

Debergh, P.C. & Read, P.E.1991. Micropropagation. In: P.C. Debergh & R.H. Zimmerman (eds) Micropropagation Technology and Application. Kluwer Academic Publishers. Netherlands. pp 1-13

Debergh, P. & Vanderschaeghe, A. 1988. Some symptoms indicating the presence of bacterial contaminants in plant tissue culture. *Acta Horticulturae*. **225**:77-81

Debergh, P. & Vanderschaeghe, A. 1990. Mass propagation of *in vitro* plantlets. *Chronica Horticulturae*. **30(1)**:1-2

Debergh, P.C., Harbaoui, Y. & Lemuer, R. 1981. Mass propagation of globe artichoke (*Cynara scolymus*): Evaluation of different hypotheses to overcome vitrification with special reference to water potential. *Physiologia Plantarum.* **53**:181-187

Debergh, P.C., Topoonyanont, N., Van Huylenbroeck, J., Moreira da Silva, H. & Oyaert, E. 2000. Preparation of micro plants for *ex vitro* establishment. *Acta Horticulturae.***530**:269-275

De Fossard, R.A., & De Fossard, H. 1988. Coping with microbial contaminants and other matters in a small commercial micropropagation laboratory. *Acta Horticulturae.* **225:**167-176

De Klerk, G.J. 2000. Rooting treatment and the *ex vitrum* performance of micropropagated plants. *Acta Horticulturae.* **530**:277-289

Dell, B. 1996. Diagnosis of nutrient deficiencies in *Eucalyptus*. In: P.M. Attiwill & M.A. Adams (eds). Nutrition of *Eucalyptus*. CSIRO Publishing. Collingwood. pp 417-440

Denison, N.P. 1999. Mondi Forests Tree Improvement Research: (1968-1998) 30 years in perspective. Internal Report, Mondi Forests. pp 28-39

Denison, N.P. & Kietzka, J.E. 1993. The use and importance of hybrid intensive forestry in South Africa. *South African Forestry Journal.* **165**:55-60

De Riek, J. Van Cleemput, O. & Debergh, P.C. 1991. Carbon metabolism of micro propagated *Rosa multiflora* L. *In Vitro Cellular and Developmental Biology - Plant.* **27**:57-63

Desjardins, Y. Hdider, C & de Riek, J. 1995. Carbon nutrition *in vitro.* Regulation and manipulation of carbon assimilation in micropropagated systems. In: J. Aitken-Christie, T. Kozai & M.A.L. Smith (eds). Automation and Environmental Control in Plant Tissue Culture. Kluwer Academic Publishers, Netherlands. pp 441-470

Dey, S. 2002. Cost-effective mass cloning of plants in liquid media using a novel Growtek bioreactor. First international symposium on liquid systems for *in vitro* mass propagation of plants. Cost 843 working group. Norway. pp 69

Dodds J.H., & Roberts, L.W. 1985. Experiments in Plant Tissue Culture, second edition. Cambridge University Press, Cambridge. pp 27

Donnelly, D.J. & Tisdall, L. 1993. Acclimatization strategies for micropropagated plants. In: M.R. Ahuja (ed). Micropropagation of Woody Plants. Kluwer Academic Publishers, Netherlands. pp 153-166

Duchefa Catalogue 1998-1999. Biochemicals, plant cell and tissue culture, plant molecular biochemical. Netherlands. pp 9-21

Ellis, D.D. & Webb, D.T. 1993. Light regimes used in conifer tissue culture. In: M.R. Ahuja (ed). Micropropagation of Woody Plants. Kluwer Academic Publishers, Netherlands. pp 31-55

Errebhi, M. & Wilcox, C.A. 1990. Plant species response to ammonium-nitrate concentration ratios. *Journal of Plant Nutrition.* **13**:1017-1029

Escalona, M., Lorenzo, J.C., González, B., Daquinta, M., González, J.L., Desjardins, Y. & Borroto, C.G. 1999. Pineapple (*Ananas comosus* L. Merr) micropropagation in temporary immersion systems. *Plant Cell Reports.* **18:**743-748

Etienne, H. 2000. Direct sowing of temporary immersion-produced somatic embryos. *Agricell Report*. **34(3)**:17-18

Etienne, H., Lartaud, M., Michaux-Ferrière, N., Carron, M.P., Berthouly, M. & Teisson, C. 1997. Improvement of somatic embryogenesis in *Hevea brasiliensis* (Müll.Arg) using the temporary immersion techniques. *In Vitro Cellular and Developmental Biology - Plant.* **33**:81-87

Falkiner, F.R. 1997. Antibiotics in plant tissue culture and micro-propagation - what are we aiming at? In: A.C. Cassells (ed). Pathogen and Microbial Contamination Management in Micropropagation. Kluwer Academic Publishers, Netherlands. pp 155-160

Falkiner, F.R. 2000. Antibiotics and antibiotic resistance associated with plants, fruits and vegetables. *Acta Horticulturae.* **530**:83-91

Fowler, M.W. 1988. Problems in commercial exploitation of plant cell cultures. In: unknown (eds). Applications of Plant Cell and Tissue Culture. John Wiley & Sons, New York. pp 239-253

Fujiwara, K. & Kozai, T. 1995. Physical micro-environment and its effects. In: J. Aitken-Christie, T. Kozai & M.A.L. Smith (eds). Automation and Environmental Control in Plant Tissue Culture. Kluwer Academic Publishers, Netherlands. pp 319-369

Fukui, H. & Tanaka, M. 1995. An envelope-shaped film culture vessel for plant cell suspension cultures and metabolite production without agitation. *Plant Cell, Tissue and Organ Culture.* **41**:17-21

Fuller, M.P. & Pizzey, T. 2001. Teaching fast and reliable plant tissue culture using PPM and *Brassicas*. *Acta Horticulturae*. **560**:567-569

Gamborg, O.L. 1970. The culture of plants with ammonium salts as the sole nitrogen source. *Plant Physiology.* **45**:598-600

Gamborg, O.L. & Shyluk, J.P. 1981. Nutrition, media and characteristics of plant cell and tissue culture. In: T.A. Thorpe (ed). Plant Tissue Culture Methods and Applications in Agriculture. Academic Press. New York. pp 21-44

Gawel, N.J. & Robacker, C.D. 1990. Somatic embryogenesis in two *Gossypium hirsutum* genotypes on semi-solid versus liquid proliferation media. *Plant Cell, Tissue and Organ Culture.* **23**:201-204

George, E.F. 1993. Plant propagation by tissue culture. The technology. Exegetics Ltd. Edington. **1**:612-635

George, E.F. Puttock, D.J.M. & George, H.J. 1987. Plant culture media. Vol 1. Formulations and Uses. Exegetics Limited. UK.

George, E.F. Puttock, D.J.M. & George, H.J. 1988. Plant culture media. Vol 2. Commentary and Analysis. Exegetics Limited. UK.

Gislerød, H.R. & Selliah, R. 2002. Relation between macro- and micronutrient fertilization in greenhouse production and microhydroponic systems. First international symposium for *in vitro* mass propagation of plants. Cost 843 working group. Norway. pp 54-55

González, E.J. 2002. Mass propagation of tropical crops in temporary immersion systems: present status and future prospects. First international symposium for *in vitro* mass propagation of plants. Cost 843 working group. Norway. pp 44-45

Grigoriadou, K., Vasilakakis, M. & Elefteriou, E.P. 2002. Effect of temperature and liquid medium on olive microshoot development using a novel temporary immersion system. First international symposium for *in vitro* mass propagation of plants. Cost 843 working group. Norway. pp 106-107

Grönroos, R. Von Arnold, S. 1987. Initiation of roots on hypocotyl cuttings of *Pinus contorta in vitro*. *Physiologia Plantarum* **69**:227-236

Gupta, P.K. 2002. Mass propagation of conifer trees in liquid cultures - possibilities, pitfalls and bottlenecks. First international symposium on liquid systems for *in vitro* mass propagation of plants. Cost 843 working group. Norway. pp 16

Gupta, P.K. & Mascarenhas, A.F. 1983. Essential oil production in relation to organogenesis in tissue cultures of *Eucalyptus citriodora* Hook. In: S.K. Sen & K.L. Giles (eds). Plant Cell Culture in Crop Improvement. Plenum Press, New York. pp 299-308

Hackett, W.P. & Murray, J.R. 1993. Maturation and rejuvenation in woody species. In: M.R. Ahuja (ed). Micropropagation of Woody Plants. Kluwer Academic Publishers, Netherlands. pp 93-105

Hahn, E-J. & Paek, K-Y. 2002. Plantlet growth and CO_2 concentration in the culture vessel as affected by medium supply in bioreactor culture of *Chrysanthemum.* First international symposium for *in vitro* mass propagation of plants. Cost 843 working group. Norway. pp 25

Hammatt, N. 1992. Progress in the biotechnology of trees. *World Journal of Microbiology and Biotechnology.* **8**:369-377

Harrell, R.C., Bieniek, M., Hood, C.F., Munilla, R. & Cantliffe, D.J. 1994. Automated, *in vitro* harvest of somatic embryos. *Plant Cell, Tissue and Organ Culture.* **39**:171-183

Harris, R.E. & Mason, E.B.B. 1983. Two machines for *in vitro* propagation of plants in liquid media. *Canadian Journal of Plant Science.* **63**:311-316

Hassall, K.A. 1990. Non-systemic organic fungicides. In: K.A. Hassall (ed). The Biochemistry and Uses of Pesticides. Second Edition. Macmillan. pp 286-314

Hayashi, M., Fujita, N., Kitaya, Y. & Kozai, T. 1992. Effects of sideward lighting on the growth of potato plantlets *in vitro. Acta Horticulturae.* **319**:163-166

Heyerdahl, P.H., Olsen, O.A.S. & Hvoslef-Eide, A.K. 1995. Engineering aspects of plant propagation in bioreactors. In: J. Aitken-Christie, T. Kozai & M.A.L. Smith (eds). Automation and Environmental Control in Plant Tissue Culture. Kluwer Academic Publishers, Netherlands. pp 87-124

Hills, W.E. & Brown, A.G. 1978. *Eucalyptus* for wood production. CSIRO, Australia. pp 434

Hohe, A., Winkelmann, T. & Schwenkel, H.G. 1999. The effect of oxygen partial pressure in bioreactors on cell proliferation and subsequent differentiation of somatic embryos of *Cyclamen persicum*. *Plant Cell, Tissue and Organ Culture*. **59**:39-45

Holden, M.A. & Yeoman, M.M. 1987. Optimization of product yield in immobilized plant cell cultures. In: G.W. Moody & P.B. Baker (eds). Bioreactors and Biotransformations. Elsevier Applied Science Publishers LT. Essex. pp 1-11

Holdgate, D.P. & Zandvoort, E.A. 1997. Strategic considerations for the establishment of micro-organism-free tissue cultures for commercial ornamental micropropagation. In: A.C. Cassells (ed). Pathogen and Microbial Contamination Management in Micropropagation. Kluwer Academic Publishers, Netherlands. pp 15-22

Hong, Y.C., Labuza, T.P. & Harlander, S.K. 1989. Growth Kinetics of Strawberry cell Suspension Cultures in Shake Flask, Airlift, Stirred-Jar, and Roller Bottle Bioreactors. *Biotechnology Progress*. **5(4):**137-143

Horgan, K. & Holland, L. 1989. Rooting micropropagated shoots from mature *Radiata* pine. *Canadian Journal of Forest Research*. **19**:1309-1315

Hussain, S., Lane, S.D. & Price, D.N. 1994. A preliminary evaluation of the use of microbial culture filtrates for the control of contaminants in plant tissue culture systems. *Plant Cell, Tissue and Organ Culture*. **36**:45-51

Hvoslef-Eide, A.K., Olsen, O.A.S., Lyngved, R. & Heyerdahl, P.H. 2002. Bioreactor design for clonal propagation of somatic embryos. First international symposium for *in vitro* mass propagation of plants. Cost 843 working group. Norway. pp 71-73

Ibaraki, K., Iida, Y. & Kurata, K. 1992. Effects of air currents on gas exchange of culture vessels. *Acta Horticulturae*. **319**:221-224

Ikemori, Y.K. 1987. Epicormic shoots from the branches of *Eucalyptus grandis* as an explant source for *in vitro* culture. *Commonwealth Forestry Review.* **44(4)**:351-356

Ingram, B. & Mavituna, F. 2000. Effect of bioreactor configuration on the growth and maturation of *Picea sitchensis* somatic embryo cultures. *Plant Cell, Tissue and Organ Culture.* **61**:87-96

Jackson, M.B. 2002. Ventilation of plant tissue cultures. First international symposium for *in vitro* mass propagation of plants. Cost 843 working group. Norway. pp 56-57

Jackson, M.B., Abbot, A.J., Belcher, A.R., Hall, K.C., Butler, R. & Cameron, J. 1991. Ventilation in plant tissue cultures and effects of poor aeration on ethylene and carbon dioxide accumulation, oxygen depletion and explant deployment. *Annals of Botany.* **67**:229-237

Jiménez, E., Pérez, N., de Feria, M., Barbón, R., Capote, A., Chávez, M., Quiala, E. & Pérez, J.C. 1999. Improved production of potato microtubers using a temporary immersion system. *Plant Cell, Tissue and Organ Culture.* **59**:19-23

Jones, O.P. 1993. Propagation of apple *in vitro*. In: M.R. Ahuja (ed). Micropropagation of Woody Plants. Kluwer Academic Publishers, Netherlands. pp 169-186

Jones, A.M. & Petolino, J.F. 1988. Effects of support medium on embryo and plant production from cultured anthers of soft-red winter wheat (*Triticum aestivum* L.). *Plant Cell, Tissue and Organ Culture*. **12**:253-261

Katznelson, H. & Sutton, M.D. 1951. Inhibition of plant pathogenic bacteria *in vitro* by antibiotics and quaternary ammonium compounds. *Canadian Journal of Botany.* **29:**270-278

Kevers, C., Coumans, M., Coumans-Gillès, M-F. & Gaspar, T. 1984. Physiological and biochemical events leading to vitrification of plants cultured *in vitro*. *Physiologia Plantarum.* **61**:69-74

Khan, P.S.S.V., Kozai, T. Nguyen, Q.T., Kubota, C. & Dhawan, V. 2002. Growth and net photosynthetic rates of *Eucalyptus tereticornis* Smith under photomixotrophic and various photoautotrophic micropropagation conditions. *Plant Cell, Tissue and Organ Culture*. **71**:141-146.

Kirschbaum, M.U.F. 1991. Effects of photon flux density on nutrient productivity in *Eucalyptus grandis* seedlings. *Australian Journal of Plant Physiology*. **18**:307-315

Kneifel, W., & Leonhardt, W. 1992. Testing different antibiotics against gram-positive and gram-negative bacteria isolated form plant tissue culture. *Plant Cell, Tissue and Organ Culture*. **29**:139-144

Kokko, H., Häikiö, E. & Kärenlampi, S. 2002. Propagation of hybrid Aspen in temporary immersion system. First international symposium for *in vitro* mass propagation of plants. Cost 843 working group. Norway. pp 104-105

Kosky, R.G., Perozo, J.V., Valero, N.A. & Peñalver, D.A. 2002. Somatic embryo germination of *Psidium guajava* L. cv. Cuban Red Dwarf in temporary immersion system. First international symposium on liquid systems for *in vitro* mass propagation of plants. Cost 843 working group. Norway. Poster

Kozai, T. 1988. Autotrophic (sugar free) tissue culture for promoting the growth of plantlets *in vitro* and for reducing biological contamination. Proceedings of international symposium on application of biotechnology for small industries development in developing countries. Thailand. pp 189 –209

Kozai, T. & Smith, M.A.L. 1995. Environmental Control in plant tissue culture. General introduction and overview. In: J. Aitken-Christie, T. Kozai & M.A.L. Smith (eds). Automation and Environmental Control in Plant Tissue Culture. Kluwer Academic Publishers, Netherlands. pp 301-318

Kubota, C. & Kozai, T. 1992. Growth and net photosynthetic rate for *Solanum tuberosum in vitro* under forced and natural ventilation. *HortScience* **27**:1312-1314

Kumar, P.P, Reid, D.M. & Thorpe, T.A. 1987. The role of ethylene and carbon dioxide in differentiation of shoot buds in excised cotyledons of *Pinus radiata in vitro. Physiologia Plantarum.* **69**:244-252

Kumar, P.P., Joy (IV), R.W. & Thorpe, T.A. 1989. Ethylene and carbon dioxide accumulation, and growth of cell suspension cultures of *Picea glauca* (White Spruce). *Journal of Plant Physiology.* **135**:592-596

Kunneman, B.P.A.M. & Faaij-Groenen, G.P.M. 1988. Elimination of bacterial contaminants: A matter of detection and transplanting procedures. *Acta Horticulturae.* **225**:183-188

Kurata, K. 1995. Automated systems for organogenesis. In: J. Aitken-Christie, T. Kozai & M.A.L. Smith (eds). Automation and Environmental Control in Plant Tissue Culture. Kluwer Academic Publishers, Netherlands. pp 301-318

Lakshmi Sita, G. 1993. Micropropagation of *Eucalyptus.* In: M.R. Ahuja (ed). Micropropagation of Woody Plants. Kluwer Academic publishers, Netherlands. pp 263-280

Leifert, C. 2000. Quality assurance systems for plant cell and tissue culture: The problem of latent persistence of bacterial pathogens and *Agrobacterium* based transformation vector system. *Acta Horticulturae.* **530**:87-92

Leifert, C. & Waites, W.M. 1994. Dealing with microbial contaminants in plant tissue culture: Hazard analysis and critical control points. In: P.J. Lumsden, J.R. Nicholas & W.J. Davies (eds). Physiology, growth, and development of plants in tissue culture. Kluwer Academic Publishers. Wageningen. pp 363-378

Leifert, C. & Woodward, S. 1998. Laboratory contamination management: The requirement for microbiological quality assurance. *Plant Cell, Tissue and Organ Culture.***52**:83-88

Leifert, C., Waites, W.M. & Nicholas, J.R. 1989. Bacterial contaminants of micro propagated plant cultures. *Journal of Applied Bacteriology.* **67**:353-361

Le Roux, J.J. & Van Staden, J. 1991. Micropropagation and tissue culture of *Eucalyptus*: A review. *Tree Physiology* **9**:435-477

Lorenzo, J.C., González, L.B., Escalona, M., Teisson, C., Espinosa, P. & Borroto, C. 1998. Sugarcane shoot formation in an improved temporary immersion system. *Plant Cell, Tissue and Organ Culture.* **54**:197-200

Mackay, W.A. & Kitto, S.L. 1988. Factors affecting *in vitro* shoot proliferation of French tarragon. *Journal of the American Society of Horticultural Science.* **113**:282-287

Maene, L. & Debergh, P. 1985. Liquid medium additions to established tissue cultures to improve elongation and rooting *in vivo*. *Plant Cell, Tissue and Organ Culture.* **5**:23-33

Majada, J.P. 1998. Control of hyperhydricity in liquid micropropagation systems. *Agricell Report.* **30(4)**:1

Martre, P., Dominique, L., Just, D. & Teisson, C. 2001. Physiological effects of temporary immersion on *Hevea brasiliensis* callus. *Plant Cell, Tissue and Organ Culture.* **67:**25-35

Mascarenhas, A.F. & Muralidharan, E.M. 1989. Tissue culture of forest trees in India. *Current Science*. **58(11)**:606-613

Matthys, D., Gielis, J. & Debergh, P. 1995. Ethylene. In: J. Aitken-Christie, T. Kozai & M.A.L. Smith (eds). Automation and Environmental Control in Plant Tissue Culture. Kluwer Academic Publishers, Netherlands. pp 473-491

McClelland, M.T. & Smith, M.A.L. 1990. Influence of type, closure and explant orientation on *in vitro* performance of five woody species. *HortScience* **25**:797-800

McComb, J.A. & Bennett, I.J. 1986. Eucalypts (*Eucalyptus* spp.) In: Y.P.S. Bajaj (ed). Biotechnology in Agricultue and Forestry. Vol. 1: Trees 1. Springler-Verlag. Berlin. pp 340-362

Minocha, S.C. 1980. Cell and tissue culture in the propagation of forest trees. In: F. Sala, B. Parisi, R. Cella & O. Ciferri (eds). Plant Cell Cultures: Results and Perspectives. Elsevier/North-Holland Biomedical Press. Holland. pp 295-300

Monette, P. 1986. Micropropagation of kiwifruit using non-axenic shoot tips. *Plant Cell, Tissue and Organ Culture*. **6**:73-82

Morris, P., Scragg, A.H., Smart, N.J., & Stafford, A. 1985. Secondary product formation by cell suspension cultures. In: R.A. Dixon (ed). Plant Cell Culture: A Practical Approach. IRL press. Oxford. pp127-167

Murashige, T. & Skoog, F. 1962. A revised medium for rapid growth and bio assays with tobacco tissue cultures. *Physiologia Plantarum.* **15**:473-496

Osmotek Life Line Products. 1998. (25/8/1998). http://www.osmotek.com/product.htm.

Owen, H.R., Wengerd, D. & Miller, A.R. 1991. Culture medium pH is influenced by basal medium, carbohydrate source, gelling agent, activated charcoal, and medium storage method. *Plant Cell Reports* **10**:583-586

Owen, D.L., Van der Zel, D.W. 2000. Trees, forests and plantations in Southern Africa. In: D.L. Owen (ed) South African Forestry Handbook. V & R Printers, Pretoria. Pp 3-8

Paek, K.Y., Hwang, J.K. & Han, B.H. 1993. Perspective of handicaps for commercial application of micropropagation in Korea. In: W.Y. Soh, J. R. Liu & A. Komamine (eds), Advances in Developmental Biology and Biotechnology of Higher Plants. The Korean Society of Plant Tissue Culture, Korea. pp 38-70

Paek, K.Y. & Hahn, E-J. 2002. Application of bioreactor system for scale up production of horticultural and medicinal plants. First international symposium on liquid systems for *in vitro* mass propagation of plants. Cost 843 working group. Norway. pp 20-21.

Pamfil, D. 2002. Comparison of the formation of *Cymbidium* protocorms and plantlets on agar-solidified and liquid mediums (stationary, agitated and bioreactor). First international symposium on liquid systems for *in vitro* mass propagation of plants. Cost 843 working group. Norway. pp 120-121

Phan, C.T. 1991. Vitreous state *in vitro* culture: ethylene *versus* cytokinin. *Plant Cell Reports.* **9**:517-519.

Phillips, R., Arnott, S.M. & Kaplan, S.E. 1981. Antibiotics in plant tissue culture: Rifampicin effectively controls bacterial contaminants without affecting the growth of short-term explants cultures of *Helianthus tuberosus. Plant Science Letters.* **21**:235-240

Pinker, I. 2000. Characterization of stem quality in relation to rooting process in *Prunus* and *Amelanchier* cultures. *Acta Horticulturae.* **530**:387-395

Preece, J.E. 1995. Can nutrient salts partially substitute for plant growth regulators? *Plant Tissue Culture and Biotechnology.* **1(1):**26-36

Preece, J.E. & Sutter, E.G. 1991. Acclimatization of micropropagated plants to the greenhouse and field. In: P.C. Debergh & R.H. Zimmerman (eds). Micropropagation. Kluwer Academic Publishers. Netherlands. pp 71-93

Preil, W. 1991. Application of bioreactors in plant propagation. In: P.C. Debergh & R.H. Zimmerman (eds). Micropropagation. Kluwer Academic Publishers. Netherlands. pp 425-445

Preil, W. & Hempfling, T. 2002. Application of temporary immersion system in propagation of *Phalaenopsis*. First international symposium on liquid systems for *in vitro* mass propagation of plants. Cost 843 working group. Norway. pp 47-48

Preil, W., Florek, P., Wix, U. & Beck, A. 1988. Towards mass propagation by use of bioreactors. *Acta Horticulturae*. **226**:99-105

Reed, B.M. & Tanprasert, P. 1995. Detection and control of bacterial contaminants of plant tissue cultures. A review of recent literature. *Plant Tissue Culture and Biotechnology*. **1(3)**:137-142

Reed, B.M., Mentzer, J., Tanprasert, P. & Yu, X. 1997. Internal bacterial contamination of micropropagated hazelnut: Identification and antibiotic treatment. In: A.C. Cassells (ed). Pathogen and Microbial Contamination Management in Micropropagation. Kluwer Academic Publishers, Netherlands. pp 169-174

Sage, D.O. & Schroeder, M.B. 2002. Growth and development of nodular callus of *Narcissus pseudonarcissus* cvs in 'RITA' temporary immersion bioreactor. First international symposium on liquid systems for *in vitro* mass propagation of plants. Cost 843 working group. Norway. pp 112-113

Sandal, I., Bhattacharya, A. & Ahuja P.S. 2001. An efficient liquid culture system for tea shoot proliferation. *Plant Cell, Tissue and Organ culture*. **65:** 75-80

Seon, J.H., Kim, Y.S., Son, S.H. & Paek, K.Y. 2000. The feed-batch culture system using bioreactor for the bulblet production of oriental lilies. *Acta Horticulturae*. **520**:53-59

Shields, R., Robinson, S.J. & Anslow, P.A. 1984. Use of fungicides in plant tissue culture. *Plant Cell Reports*. **3**:33-36

SIGMA Catalogue. 1994. SIGMA plant culture catalogue. Sigma-Aldrich Corporation. United Kingdom. pp 39

Simonton, W., Robacker, C. & Krueger, S. 1991. A programmable micropropagation apparatus using cycled liquid medium. *Plant Cell, Tissue and Organ Culture*. **27**:211-218

Skidmore, D.I., Simons, A.J. & Bedi, S. 1988. *In vitro* culture of shoots of *Pinus caribaea* on liquid medium. *Plant Cell, Tissue and Organ Culture.* **14**:129-136

Skirvin, R.M., Abu-Qaoud, H., Sriskandarajah, S. & Harry, D.E. 1993. Genetics of micropropagated woody plants. In: M.R. Ahuja (ed). Micropropagation of Woody Plants. Kluwer Academic Publishers, Netherlands. pp 121-152

Smith, M.R., 1996. *Eucalyptus* takes on "super tree" status. In: M.R. Smith (ed). Southern Hemisphere Forest Industry Journal. Trade and media Services LTD. New Zealand. pp 1-16

Smith, M.K. & Drew, R.A. 1990. Current applications of tissue culture in plant propagation and improvement. *Australian Journal of Plant Physiology.* **17:**267-289

Smith, M.A.L. & McClelland, T. 1991. Gauging the quality and performance of woody plants produced *in vitro*. *In Vitro Cellular and Developmental Biology*. **27**:52-56

Smith, M.A.L. & Spomer, L.A. 1995. Vessels, gels, liquid media and support systems. In: J. Aitken-Christie, T. Kozai & M.A.L. Smith (eds). Automation and Environmental Control in Plant Tissue Culture. Kluwer Academic Publishers, Netherlands. pp 371-404

Sommer, H.E. 1981. *In vitro* methods applied to Forest trees. In: T.A. Thorpe. Plant Tissue Culture Methods and Applications in Agriculture. Kluwer Academic Press. Netherlands. pp 349-358

Stasinopoulos, T.C. & Hangarter, R.P. 1990. Preventing photochemistry in culture media by long-pass light filters which alters growth of cultured tissues. *Plant Physiology.* **93**:1365-1369.

Styer, D.J. 1985. Bioreactor technology for plant propagation In: R.R. Henke, K.W. Hughes, M.J. Constantin and A. Hollanender (eds) Tissue Culture in Forestry and Agriculture. Plenum Press. New York. pp 117-130

Svensson, M. 2000. Effect of irradiance level during *in vitro* propagation of *Aristolochia manchuriensis*. *Acta Horticulturae*. **530**:403-408

Takayama, S. 2002. Practical aspects of bioreactor application in mass propagation. First international symposium on liquid systems for *in vitro* mass propagation of plants. Cost 843 working group. Norway. pp 66-68

Takayama, S. & Akita, M. 1994. The types of bioreactors used for shoots and embryos. *Plant Cell, Tissue and Organ Culture*. **39**:147-156

Takayama, S. & Akita, M. 1998. Bioreactor techniques for large-scale culture of plant propagules. *Advanced Horticultural Science*. **12**:93-100

Tanaka, K., Fujiwara, K. & Kozai, T. 1992. Effects of relative humidity in the culture vessel on the transpiration and net photosynthetic rates of potato plantlet *in vitro*. *Acta Horticulturae*. **319**:59-64

Tanimoto, S. & Ishioka, N. 1991. Effects of gelling agents on adventitious organ induction and callus growth in some plant species. *Bulletin of the Faculty of Agriculture, Saga University*.**70**:61-66

Teisson, C. 1998. Temporary Immersion Bioreactor systems. *Agricell Report*. **31(4)**:30

Teisson, C. & Alvard, D. 1998. *In vitro* propagation of potato micro tubers in liquid medium using the temporary immersion system. Conference on potato seed production by tissue culture. Brussels. Cost 822. European Commission.

Teisson, C. & Seon, J.H. 1999. Temporary-immersion micropropagation of coffee and lily. *Agricell Report*. **32(1)**:2

Teisson, C., Alvard, D., Berthouly, B., Cote, F., Excalant, J.V. Etienne, H. & Lartaud, M. 1996. Simple apparatus to perform plant tissue culture by temporary immersion. *Acta Horticulturae.* **440**:521-526.

Teng, W.L. & Nicholson, L. 1997. Antibiotic pulse treatments disinfect root explants. *Agricell Report.* **28(6)**:41

Termignoni, R.R., Wang, P-J. & Hu C-Y. 1996. Somatic embryo induction in *Eucalyptus dunnii. Plant Cell, Tissue and Organ Culture.* **45:**129-132

Thompson, M.R. & Thorpe, T.A. 1987. Metabolic and non-metabolic roles of carbohydrates. In: J.M. Bonga, & D.J. Durzan (eds). Cell and Tissue Culture in Forestry. Volume 1. General Principals and Biotechnology. Martinus Nijhoff, Dordrecht. pp 89-112

Thorpe, T.A., Harry, I.S. & Kumar, P.P. 1991. Application of micropropagation in forestry. In: P.C. Debergh & R.H. Zimmerman (eds). Micropropagation Technology and Application. Kluwer Academic Publishers, Netherlands. pp 311-336

Thorpe, T.A. & Kumar, P.P. 1993. Cellular control of morphogenesis. In: M.R. Ahuja (ed). Micropropagation of Woody Plants. Kluwer Academic Publishers, Netherlands. pp 11-29

Tisserat, B. & Vandercook, C.E. 1985. Development of an automated plant culture system. *Plant Cell, Tissue and Organ Culture.* **5**:107-117

Trindade, H., Ferreira, J. & Pais, M.S. 1990. *In vitro* rooting of *Eucalyptus globulus* Labill. Abstracts of VII International Congress of Plant Cell Culture. Amsterdam. pp 138

Turnbull, J.W. 1991. Future use of *Eucalyptus:* opportunities and problems. In: A.P.G. Schönau (ed.). Symposium on Intensive Forestry: the Role of *Eucalyptus.* IUFRO Proceedings, Vol. 1. 2-6 September. South Africa. pp 2-27

Uosukainen, M., Rantala, S., Manninen, A. & Vestberg, M. 2000. Improvement of microplant establishment through *in vitro* and *ex vitro* exogenous chemical applications. *Acta Horticulturae.* **530**:325-331

Van Telgen, H.-J., Van Mil, A. & Kunneman, B. 1992. Effect of propagation and rooting conditions on acclimatization of micropropagated plants. *Acta Botanical Neerland.* **41(4)**:453-459

Villalobos, V.M., Leung, D.W.M. & Thorpe, T.A. 1984. Light-cytokinin interaction in shoot formation in cultured cotyledon explants of *radiata* pine. *Physiologia Plantarum.* **61**:497-504

Viss, P.R., Brooks, E.M. & Driver, J.A. 1991. Technical note: A simplified method for the control of bacterial contamination in woody plant tissue culture. *In Vitro Cellular Developmental Biology.* **27**:42

VITROPIC CIRAD pamphlet 2000. RITA® Temporary Immersion System for Plant Tissue culture. http://www.cirad.fr

Von Arnold, S. 1987. Effect of sucrose on starch accumulation in adventitious bud formation on embryos of *Picea abies. Annals of Botany.* **59**:15-22

Von Arnold, S. & Eriksson, T. 1984. Effect of agar concentration on growth and anatomy of adventitious shoots of *Picea abies* (L.) Karst. *Plant Cell, Tissue and Organ Culture.* **3**:257-264

Walker, P.N. 1995. System analysis and engineering. In: J. Aitken-Christie, T. Kozai & M.A.L. Smith (eds). Automation and Environmental Control in Plant Tissue Culture. Kluwer Academic Publishers, Netherlands. pp 65-85

Warrag, E.I., Lesney, M.S. & Rockwood, D.L. 1990. Micropropagation of field tested superior *Eucalyptus grandis* hybrids. *New Forests.* **4**:67-79.

Watt, M.P., Gauntlett, B.A. & Blakeway, F.C. 1996. Effect of anti-fungal agents on *in vitro* cultures of *Eucalyptus grandis*. *Suid-Afrikaanse Bosboutydskrif.* **175**:23-27

Watt, M.P., Blakeway, F.C., Cresswell, F.C. & Herman, B. 1991. Somatic embryogenesis in *Eucalyptus grandis*. *Suid-Afrikaanse Bosboutydskrif* **157:**59-65

Watt, M.P., Blakeway, F.C., Herman, B. & Denison, N. 1997a. Biotechnology developments in tree improvement programmes in commercial forestry in South Africa. *South African Journal of Science*. **93**:100-103

Watt, M.P., Blakeway, F.C., Herman, B. & Denison, N. 1997b. Developments in the use of biotechnology in commercial forestry tree improvement programmes in South Africa. Forest, biological diversity and the maintenance of the natural heritage. Protective and environmental functions of forests. Proceedings of the XI World Forestry Congress. Antalya.

Watt, M.P., Blakeway, F.C., Mokotedi, M.E.O. & Jain, S. M. 2002. Micropropagation of *Eucalyptus*. In: S.M Jain and K. Ishii (eds). Micropropagation of Woody Trees and Fruits, Kluwer Academic Publishers, Netherlands (*in press*).

Watt, M.P., Mycock, D.J., Blakeway, F.C. & Berjak, P. 2000. Applications of *in vitro* methods to *Eucalyptus* germplasm conservation. *Southern African Forestry Journal.* **187**:3-10

Watt, M.P., Duncan, E.A., Ing, M., Blakeway, F.C. & Herman, B. 1995. Field performance of micropropagated and macropropagated *Eucalyptus* hybrids. *Suid-Afrikaanse Bosboutydskrif.* **173**:17-21

Wawrosch, C., Kongbangkerd, A., Köpf, A. & Kopp, B. 2002. Shoot regeneration from nodules of *Charybdis sp.*: A comparison of semi-solid, liquid and temporary immersion culture systems. First international symposium on liquid systems for *in vitro* mass propagation of plants. Cost 843 working group. Norway. pp 110-111

Weathers, P.J., Cheetham, R.D. & Giles, K.L. 1988. Dramatic increases in shoot number and length for *Musa, Cordyline*, and *Nephrylepsis* using nutrient mists. *Acta Horticulturae.* **230**:39-44

Welander, M. Zhu, L-H. & Li, X-Y. 2002. Optimization of growing conditions for the apple rootstock M26 grown in RITA containers using temporary immersion principle. First international symposium on liquid systems for *in vitro* mass propagation of plants. Cost 843 working group. Norway. pp 102-103

Wetzstein, H.Y. & Sommer, H.E. 1983. Scanning electron microscopy of *in vitro* cultured *Liquidambar styraciflua* plantlets during acclimatization. *Journal of American Horticultural Science.* **108(3)**:475-480

Williams R.R. 1995. The chemical microenvironment. In: J. Aitken-Christie, T. Kozai & M.A.L. Smith (eds). Automation and Environmental Control in Plant Tissue Culture. Kluwer Academic Publishers, Netherlands. pp 405-440

Wilson, K.S. 1995. Commercialization of tissue culture and automated systems. In: J. Aitken-Christie, T. Kozai & M.A.L. Smith (eds). Automation and Environmental Control in Plant Tissue Culture. Kluwer Academic Publishers, Netherlands. pp 273-300

Young, P.S., Murthy H.N. & Yoeup, P.K. 2000. Mass multiplication of protocorm-like bodies using bioreactor system and subsequent plant regeneration in *Phalaenopsis. Plant Cell, Tissue and Organ Culture.* **63:**67-72

Zhu, L-H., Li, X-Y. & Welander, M. 2002. Optimization of growing conditions for the apple rootstock M26 grown in RITA containers using temporary immersion principle. First international symposium on liquid systems for *in vitro* mass propagation of plants. Cost 843 working group. Norway. pp 102-103

Zimmerman, R.H. 1997. Commercial micropropagation laboratories in the United States. In: A.C. Cassells (ed). Pathogen and Microbial Contamination Management in Micropropagation. Kluwer Academic Publishers, Netherlands. pp 39-44

Ziv, M. 1991. Vitrification: Morphological and physiological disorders of *in vitro* plants. In: P.C. Debergh & R.H. Zimmerman (eds). Micropropagation. Kluwer Academic Publishers. Netherlands. pp 45-69

Ziv, M. 1995. *In vitro* acclimatizaton. In: J. Aitken-Christie, T. Kozai & M.A.L. Smith (eds). Automation and Environmental Control in Plant Tissue Culture. Kluwer Academic Publishers, Netherlands. pp 493-516

Ziv, M. 1998. Disposable Plastic Bioreactors for Micropropagation. *Agricell Report.* **31(6)**:45

Ziv, M. 2002. Simple bioreactors for mass propagation of plants. First international symposium on liquid systems for *in vitro* mass propagation of plants. Cost 843 working group. Norway. pp 18-19

Ziv, M., Ronen, G. & Raviv, M. 1998. Proliferation of meristematic clusters in disposable pre-sterilized plastic bioreactors for the large-scale micropropagation of plants. *In Vitro Cellular and Developmental Biology - Plant.* **34**:152-158

Zobayed, S.M.A., Afreen, F. & Kozai, T. 2000. Quality biomass production via photoautotrophic micropropagation. *Acta Horticulturae.* **530**:377-385

Zobayed, S.M.A., Armstrong, J. & Armstrong W. 1999. Evaluation of a closed system, diffusive and humidity-induce convective throughflow ventilation on the growth and physiology of cauliflower *in vitro*. *Plant Cell, Tissue and Organ Culture*. **59:**113-123

Zobayed, S.M.A., Armstrong, J. & Armstrong W. 2001. Micropropagation of potato: Evaluation of closed, diffusive and forced ventilation on growth and tuberization. *Annals of Botany.* **87**: 53-59

APPENDIX 1. Pilot Study

Introduction

As reports became available on the capability of liquid bioreactor systems to improve multiplication and decrease the costs of labour, it appeared that bioreactors could be suitable for the *in vitro* culture of *Eucalyptus*. A pilot study was commenced and a liquid system was built using locally available components to verify if this was possible.

Materials and Methods

A bioreactor system was created using one-litre Schott bottles with modified lids containing an air inlet and outlet connected to pipes (Figure 1). The incoming and outgoing air was filtered through a 0.22μ filter. Tubes were connected to an aquarium air pump for the airflow and the incoming air tube, with a yellow 0-100μl pipette tip with holes punctured in it to facilitate fine bubbling aeration of the media, was suspended in the Schott bottle.

Figure 1. Schott bottles modified to make a continuous bioreactor

A comparison was undertaken with the same number of shoots being placed in semi-solid media. Coppice of GN107, GN121, GU151 and TAG31 were cut and placed into a 0.2 $g.l^{-1}$ solution of Sporgon® and the stems were then sprayed with 0.2 $g.l^{-1}$ Dithane® and left overnight. Leaves were then reduced and the stems were cut into nodes after which they were bubbled in 0.1 $g.l^{-1}$ Benlate® and 0.1 $g.l^{-1}$ Bravo® for 3 hours. The stems were sterilized with calcium hypochlorite (0.2 $g.l^{-1}$) for 5 min and Bravo (0.1 $g.l^{-1}$) for 2 minutes and rinsed with sterile distilled water. They were placed onto an initiation medium containing 0.5 $mg.l^{-1}$ of calcium pantothenate and biotin at a pH of 5.8. (Appendix 2. initiation medium - this medium was modified and optimized and used as a standard bud break medium at Mondi Forests). The axillary buds grew out and after 12 days they were excised and placed onto a growth media developed for optimal multiplication (Appendix 2. M1 medium). After a further 12 days the shoots were placed into the liquid media and a semi-solid medium as a comparison. Forty shoots per Schott bottle were placed and 40 shoots (8 shoots per jar) were placed onto semi-solid medium.

Results and Discussion

Initially there was a high contamination rate with TAG31 in the liquid system and the plants were discarded; but the plants were extremely large when they were discarded. The shoots had grown to about three times the size of those in the semi-solid media. Addition of Rifampicin 0.01 $g.l^{-1}$ to the liquid and the semi-solid media controlled the contamination. Results were taken after 18 days as the plants in the liquid system had grown to such an extent that they were no longer circulating and were becoming hyperhydric. The media in the Schott bottles had reduced to 600ml. On the whole, the plants were healthy and green and had multiplied well but a few shoots were black and a few were hyperhydric. Differences of multiplication rates of the liquid *vs.* the semi solid system can be seen in Figure 2. For the three different clones used, the liquid system gave improved multiplication compared with the semi-solid system, although each clone responded differently. Hyperhydricity occurred in the plants in the liquid system. The plants were brittle due to the complete submersion in liquid. BERTHOULY & ETIENNE (2002); PAMFIL (2002); WAWROSCH, KONGBANGKERD, KËPF & KOPP, (2002); ZIV (2002) also encountered hyperhydricity when the plants were under complete submersion in bioreactors.

Plants on the semi-solid media in jars were much smaller, and inter-nodal elongation was markedly less than those in the liquid media.

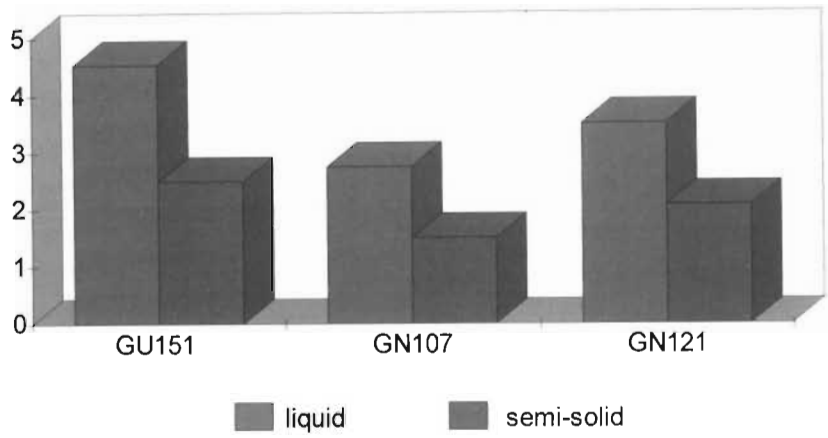


Figure 2. Average multiplication for the different clones on liquid and semi-solid media

Conclusion

If alterations could be made to this system, it would be possible to achieve higher multiplication rates with a liquid system. Although there was an increase in multiplication and a lower handling time the plants were not in a good condition. A more suitable method of liquid culture was required. There was a high loss due to contamination in this system. Problems that arose using continuous immersion were: poor airflow throughout the container; hyperhydricity; deformities and death of plants. As there were increased multiplication rates it was imperative to find a system that would counteract the problems. This lead to trials using the temporary immersion bioreactor system RITA® designed by CIRAD (CIRAD, 1999).

APPENDIX 2. Media Compositions (standard media and variations)

Murashige and Skoog (1962) medium is the standard medium used (MS). The table shows the modifications made to this medium for the different stages of growth. All media are made to a pH of 5.8, autoclaved at 121°C at a pressure of 1 Kpa.

Type of medium	Murashige & Skoog medium (MS)	Biotin ($mg.l^{-1}$)	Calcium pantothenate ($mg.l^{-1}$)	Plant growth regulators ($mg.l^{-1}$)	Gelrite ($g.l^{-1}$)	Agar ($g.l^{-1}$)	Sucrose ($g.l^{-1}$)
					In semi-solid only		
Initiation	Full strength	0.1	0.1	Kinetin 0.05 BA 0.11 NAA 0.04	2.3		25
Multiplication M1	Full strength	0.1	0.1	BA 0.2 NAA 0.01	2.3		25
½ Multiplication M2	½ strength	0.1	0.1	BA 0.2 NAA 0.01	2.3		25
M3	Full strength	0.1	0.1	BA 0.2 NAA 0.01	2.3		20
M4	Full strength	0.1	0.1	BA 0.5 NAA 0.5	2.3		20
M5	Full strength	0.1	0.1	BA 0.5 NAA 0.2	2.3		20
Elongation E1	Full strength	0.1	0.1	Kinetin 0.2 NAA 0.3 IBA 0.05	2.3		25
½ Elongation E2	½ strength	0.1	0.1	Kinetin 0.2 NAA 0.3 IBA 0.05	2.3		20
½ MS	½ strength	0.1	0.1	None		6	25
MS	Full strength	0.1	0.1	None		6	25
Rooting RM	½ strength	0.1	0.1	IBA 1		6	20