# Chapter 33

# Use of the temporary immersion bioreactor system (RITA®) for production of commercial *Eucalyptus* clones in Mondi Forests (SA)

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**Abstract**: In order to optimise tissue culture systems and to meet production targets, Mondi Forests' biotechnology programme has in the last two years concentrated efforts on the use of the RITA® temporary immersion bioreactor system. Protocols have been established for six *Eucalyptus* clones. Results indicate a four- to six-fold increase in yield, in half the time, with the RITA® system when compared with axillary bud proliferation on semi-solid media. Furthermore, plants produced from the RITA® system are hardier and acclimatize better, giving higher yields of hardened-off plants. The establishment of aseptic axillary shoots into the RITA® system is from shoots in the semi-solid system. Highest multiplication was achieved using 30-second flushes of medium every 10 minutes, starting with 50 shoots per vessel. The multiplication cycles in RITA® are between 14 and 18 days, compared with 25 to 28 days in a semi-solid system. There is minimal callus evident on the leaves and bases of the stems of plants in the RITA® system and, in addition, cold-tolerant plants have a greater rooting competence when compared with plants coming from the semi-solid system. *Ex vitro* rooting of RITA®-derived plantlets is substantially better than the plants from the semi-solid media.

*Key words*: costs, forest tree, liquid vs. semi-solid tissue culture, rooting, woody plants

*Abbreviations*: BAP - 6-benzylaminopurine; EC - electrical conductivity; IBA - indole-3-butyric acid; MS - Murashige & Skoog (1962) medium; NAA - α-naphthaleneacetic acid



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## 1. Introduction

*Eucalyptus* species and hybrids are important plantation trees throughout the world, including South Africa, as they are used for a wide variety of products. Due to diverse climatic conditions in South Africa, a variety of *Eucalyptus* species and clones is needed in order to produce appropriately site-matched planting stock in as short a time as possible. In Mondi Forests' *Eucalyptus* clonal programme, 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 and Kietzka, 1993). At present in the Company, seedlings and macro-cuttings are used to produce commercial planting clones. Additionally, *in vitro* propagation of *Eucalyptus* is used to provide stock and to replace macro-hedges and hydroponic hedges.

The latter approach is not new, as 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). To date, the most common method of micropropagation of *Eucalyptus* involves the proliferation of shoots *via* a semi-solid system (see review by Le Roux and Van Staden, 1991). While such semi-solid systems have been moderately to highly successful in terms of multiplication yields, it has become increasingly important to improve productivity and reduce the time taken to multiply commercially-important material. In the last few years, reports in the literature have shown that temporary immersion bioreactor systems, such as RITA®, have numerous advantages compared to the semi-solid methods. Temporary immersion systems combine the advantages of gelled and liquid medium, in particular having intermittent total availability of nutrients, but still allowing the plants to grow in an air space. Using RITA®, Escalona et al. (1999) found that immersion increased the multiplication rates for *in vitro* shoots of pineapple. Akula et al. (2000) reported that the immersion frequency and immersion time impacted on multiplication rates of tea. The RITA® system has been found to increase root development in *Hevea brasiliensis* (Etienne et al., 1997). Examples of other advantages listed by various authors include improved micropropagule quality, reduced consumables costs, reduced labour costs (Etienne et al., 1997; Borroto, 1998), better leaf development, reduced hyperhydricity and minimized asphyxiation of tissue (Aitken-Christie et al., 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).

This report describes how the RITA® system has been identified recently as a potentially useful method to increase multiplication yields and rooting

of *Eucalyptus* clones, at lower costs. The value of this system is discussed in terms of yields, costs and application to the *Eucalyptus* plantation component of Mondi Forests' (South Africa) tree improvement and nurseries programmes.

## 2. Materials and methods

### *2.1 Establishment of axillary buds into RITA® vessels*

The following establishment treatments were tested using six clones (GN107, GN108, NH58, GU175, GU180, TAG31) per treatment. After each treatment, explants were placed into RITA® vessels and any contamination was recorded.

Treatment a: Potted parent plants were sprayed with Sporgon® and Bravo® prior to harvesting. Single nodal explants with reduced leaf area were prepared, submerged and aerated for 3 h in 1 g $l^{-1}$ Benlate® and 1 g $l^{-1}$ boric acid. The explants were surface-sterilized with 2 g $l^{-1}$ calcium hypochlorite for 5 minutes and then placed in 0.1 g $l^{-1}$ mercuric chloride, plus two drops of Tween® 20 for two minutes. After which the explants were washed three times with sterilized water and rinsed with 1 ml $l^{-1}$ Bravo®.

Treatment b: Secondary leaders from rooted cuttings were surface-sterilized with 0.5, 1 or 2 g $l^{-1}$ calcium hypochlorite for 5, 10 or 15 min, and rinsed three times with sterile distilled water.

Treatment c: Nodal explants surface-sterilized as in treatment a), were placed onto semi-solid MS medium (Murashige and Skoog, 1962) (pH 5.8) containing kinetin (0.2µmol), NAA (0.2µmol) and BAP (0.4µmol) to enhance axillary shoot growth, and 25 g $l^{-1}$ sucrose and gelled with 2.3 g $l^{-1}$ Gelrite. Contaminant-free shoots were visually selected after two weeks.

Treatment d: Visually contaminant-free, established, multiplying *in vitro* (5 months old) shoots were selected.

Treatment e: Explants were treated as in c), after which contaminant-free shoots were selected visually and placed in 0.1 g $l^{-1}$ rifampicin® supplemented medium on a shaker at 70 rpm for seven days. Contaminant-free shoots were selected.

Treatment f: Treatment f: Visually contaminant-free multiplying *in vitro* shoots were placed in MS medium (pH of 5.8) containing 0.1 g $l^{-1}$ Rifampicin®, 25 g $l^{-1}$ sucrose shaking at 70 rpm for seven days, and contaminant-free shoots were selected thereafter.

### *2.2 Multiplication*

a) Thirty shoots were placed into RITA® vessels. Different flush times (where the plants are submerged in the media for 30 s, 1, 5 and 10 min) and rest times – (where the plants were not covered with media for 5, 10 and 20 min) were used. Multiplication was recorded after 14 days to determine which times gave the highest multiplication.
b) To test the effect, different numbers of shoots per vessels, 50, 100 and 150 shoots were placed into RITA® vessels and flushed for 30 seconds. Rest periods of 10, 20 and 30 min were used for the different numbers of shoots per vessel. Shoot multiplication rates were recorded after 21 days. MS medium (pH 5.8) with 0.8 µmol BAP, 0.01 µmol NAA, and 25 g $l^{-1}$ sucrose was used.
c) Using 50 shoots per vessel, multiplication rates and shoot size of plants, as well as electrical conductivity (EC) of media were recorded at 0, 7, 14 and 21 days. Multiple analysis of variance was undertaken.
d) Multiplication rates of semi-solid and liquid system were compared for five clones during a 21-day period. Exposure time to nutrients in the liquid system was constant throughout, a 30 seconds flush time and a 10 minute rest time was used.

### *2.3 Rooting*

Shoots from multiplication media (semi-solid and RITA®) were placed onto half-strength MS medium (pH 5.8) containing 4.9 µmol IBA and 10 g $l^{-1}$ sucrose for rooting (6 g $l^{-1}$ agar was used for the semi-solid media). After seven days in rooting medium, plants (with and without roots) were placed in the greenhouse. Rooting and survival of acclimatized plants from both systems were recorded.

## 3. Results and discussion

Establishment of nodal cuttings of six different clones directly into the RITA® vessels was the first attempt at obtaining contaminant-free cultures (Treatment a). 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 clones used (Table 1). Contamination percentages on the semi-solid medium usually ranged from 20 % to 80 % dependant on the clone and whether material was taken from pre-treated parent plants. Ikemori (1987) found the average contamination rate to be 60 % if nodal sections from 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 surface-sterilized using different concentrations of calcium hypochlorite for different periods of time. The use of the secondary leaders as explants for establishment was, however, unsuccessful (Table 1). 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 $l^{-1}$ calcium hypochlorite was used for 10 and 15 minutes, death of the shoots occurred. This was due to the fact that the secondary material is young and cannot withstand a harsh surface-sterilization regime. When using soft young material, it is difficult to obtain surface- sterilization regimes that are sufficiently rigorous to destroy the surface microbes without becoming toxic to the young shoots. 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 a concentration of disinfectant was used.

The use of the semi-solid media (Treatment c) facilitated the removal of fungal contamination, 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, bacterial contamination (average of 57 %) occurred across the clones (Table 1). When treatment d was used, where visually contaminant-free, multiplying plantlets from *in vitro* culture (for five months) were placed directly into the RITA® vessels, an average of 32 % bacterial contamination was obtained with the different clones used (Table 1).

*Table 1*: Percentage of contamination occurring when different explants were sterilized by various methods and initiated into the RITA system (shoots of 6 clones were tested)

| Sterilization treatment and explant type*) | Average % contamination (6 clones) of shoots placed into the RITA system |
|---|---|
| a | 100 |
| b | 100 |
| c | 57 |
| d | 32 |
| e | 0 |
| f | 0 |

*) see chapter 2.1

*Table 2*: 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 three sub-tropical and two cold-tolerant clones (SD - represents a mean SD)

| Clone | No. of shoots after 28 days culture on semi-solid medium | No. of shoots after 14 days culture in RITA® vessel |
|---|---|---|
| GU177 | 497 | 845 |
| GU178 | 376 | 722 |
| TAG31 | 526 | 637 |
| Average multiplication for the subtropical clones | 4.7 times (SD 0.78) | 7.3 times (SD 1.05) |
| GN107 | 187 | 237 |
| GN108 | 294 | 744 |
| Average multiplication for the cold-tolerant clones | 2.4 times (SD 0.54) | 4.9 times (SD 2.45) |

*Table 3*: Acclimatization success of shoots transfered 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)

| | Acclimatisation success (%) | | | |
|---|---|---|---|---|
| | Cultured on semi-solid medium | | Cultured in RITA® vessels | |
| Clone | Shoots with roots | Shoots without roots | Shoots with roots | Shoots 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 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. According to Phillips, et al. (1981) and Cornu and Michel (1987), Rifampicin was found to be an effective antibiotic with no phytotoxic effects to the plants. The use of 0.1 g $l^{-1}$ Rifampicin resulted in contaminant-free explants and had little effect on the shoots (Treatment e and f). All shoots without visible signs of contamination were then placed into the RITA® vessels, after which no bacterial contamination occurred (Table 1). 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. Escalona et al. (1999) and Preil and 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 and Zandvoort (1997), it is not possible to place shoots directly into the RITA® vessels without obtaining high losses.

The temporary immersion system provides a highly aerobic system for plant growth, as there is forced ventilation through the vessel lid. However the immersion times, i.e. duration or frequency, is the most decisive parameter for system efficiency (Alvard et al., 1993; Berthouly and Etienne, 2002). The flush time interval of 30 min resulted in the lowest multiplication rate. It was found that the five minute rest period gave significantly lower multiplication i.e. from 2.1 times (30 s flush) to 1.5 times at 10 minutes flush (Figure 1). 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 long or too frequent. 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 gel 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. Fujiwara and Kozai (1995) found that increasing the number of air exchanges avoided shoots becoming hyperhydric with long-term continuous liquid cultures. The 10 minute flush time caused a reduction in the multiplication. 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. 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) rates of multiplication. 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 and Hempfling (2002) found with *Phalaenopsis* that the effect of immersion frequency affected the plant growth rates and that eight immersions for 10 minutes per day were applied gave maximal 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. Matre 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 (Jimenez et al., 1999). For the *Eucalyptus* shoots, a flush time of 30 seconds with a rest period of 10 minutes gave the highest multiplication rate (3.8x).

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. 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 greatest multiplication for all three rest times tested. Starting with 50 shoots per vessel, the multiplication of the three clones tested were significantly greater using a 30-second flush every 10 or 20 minutes (2.74, 2.66x respectively) than with 100 or 150 starting shoots. The rest time of 30 minutes gave the lowest multiplication (1.3x) using 100 and 150 shoots per vessel (Figure 2). 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.

With 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 (axillary buds larger than 0.5 cm) 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). Between day 14 and 21, shoot elongation increased considerably, thus making it feasible to culture the shoots in RITA® for 21 days (Figure 4).

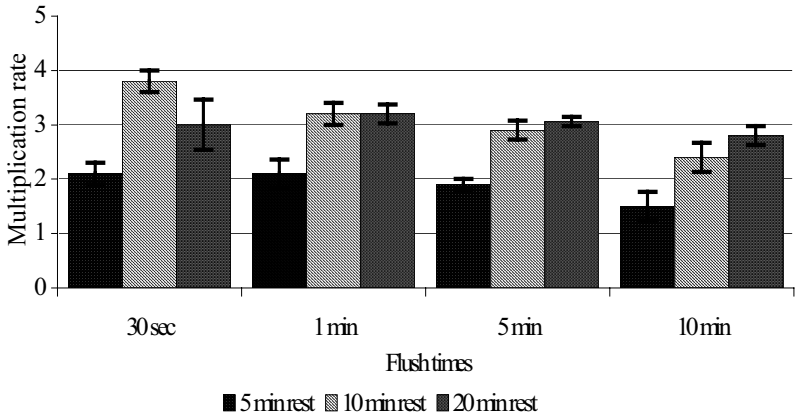


*Figure 1:* Multiplication rates of the shoots placed in the RITA® vessels at different flush vs. rest times. Each SD value represents a mean SD.

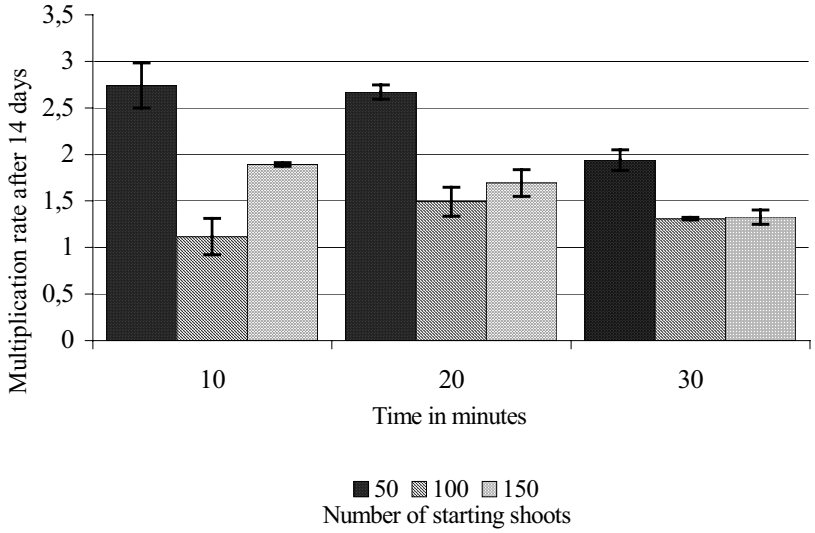


*Figure 2:* Effect of different flush intervals (10, 20 and 30 min) and shoot numbers per vessel on multiplication rates after 14 days for three clones (exposure time per flush is 30 seconds). Each SD value represents a mean SD.

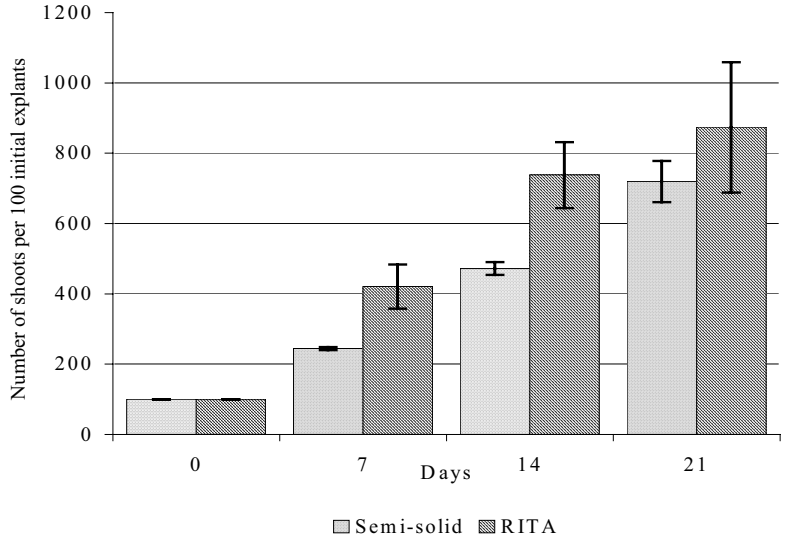


*Figure 3:* Multiplication in the RITA® and semi-solid systems (per 100 starting shoots). Each SD value represents a mean SD.

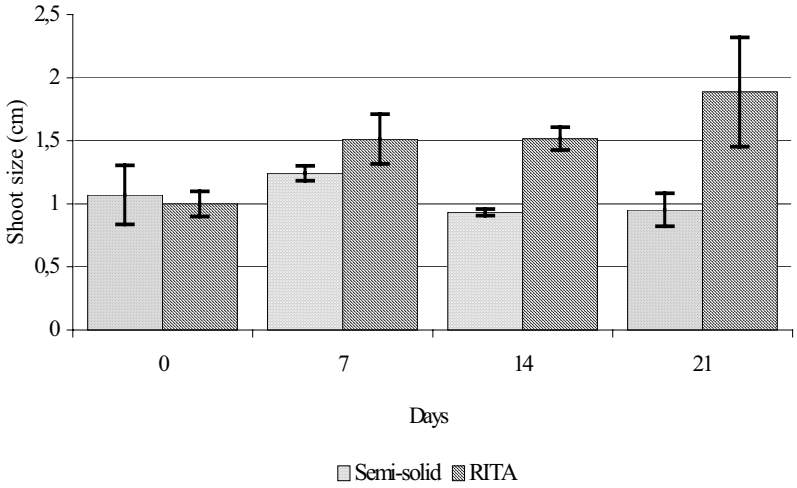


*Figure 4:* Shoot length (cm) in the RITA® and semi-solid systems (minimum of 100 shoots per system per time period). Each SD value represents a mean SD.

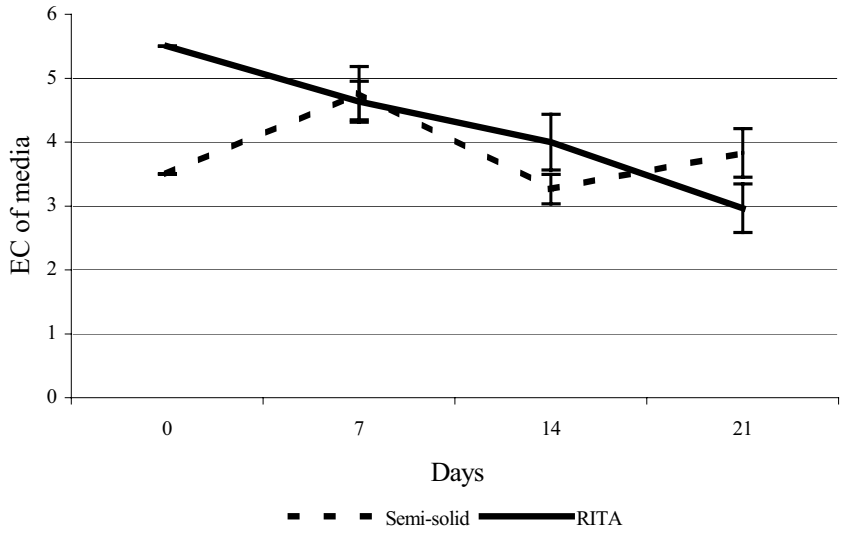


*Figure 5:* EC (μS) of media from RITA® and semi-solid systems.

The multiplication slows and elongation occurs at this time. The semi-solid system gave smaller plants and multiplication was less 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. The EC in the RITA® system was high at the beginning (5.5 μS) (Figure 5). It decreased steadily during 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 had the uptake of nutrients. 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 value. 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 that in the RITA® system. In RITA®, nutrients were immediately available. After 28 days the plants in RITA® system deteriorated which could be due to a 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.

Average multiplication rates for three sub-tropical clones and two cold-tolerant clones were calculated for the semi-solid and RITA® systems, and all clones had different multiplication rates (Table 2). All clones multiplied faster in the RITA® system compared with plants in the semi-solid system. After 28 days in the semi-solid system, subtropical clones achieved a multiplication of 4.7x while in the RITA® system the same clones achieved 7.3x during a 14 day period. The shoots of cold-tolerant clones multiplied 2.4x in the semi-solid during 28 days, and 4.9x in the RITA® system during 14 days (Figure 6). The optimum multiplication cycles in RITA® were between 14 and 21 days, whereas in the semi-solid system they were 25 to 28 days. 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 and Hempfling (2002) 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.

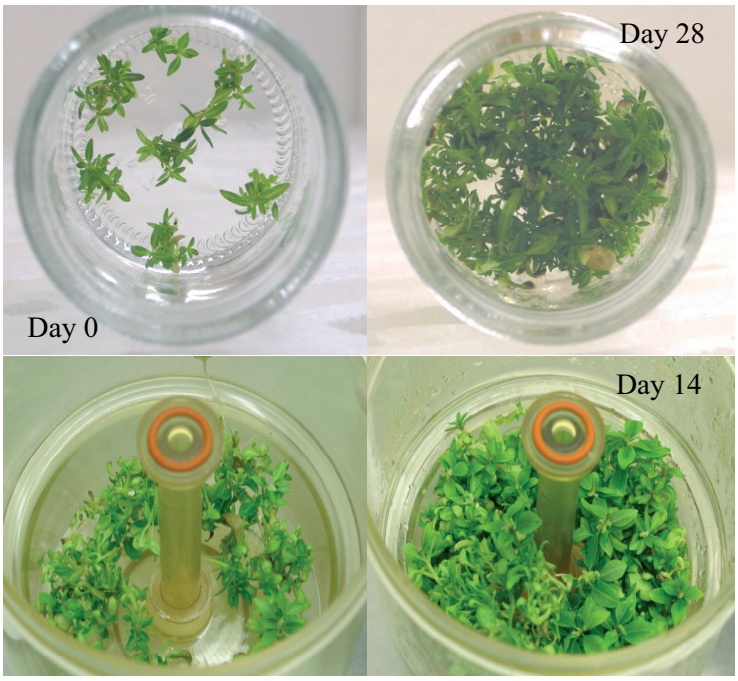


*Figure 6:* At the beginning of the cycle 8 shoots per jar (top left) and 50 shoots per RITA® (bottom left) are used, and after 28 days in the semi-solid (top right) and 14 days in the RITA® (bottom right) the multiplication which occurred is shown.

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 and Spomer, 1995). With the semi-solid system, a major barrier to tissue aeration is the enclosing of a vessel to prevent drying out and contamination. Zoybayed et al. (2001) and (Jackson, 2002) reported that sealing of culture vessels could seriously inhibit growth, development, induce hyperhydricity, reduce the leaf chlorophyll content and result in asphyxiation. According to Jackson (2002) forced ventilation allows the plants to become more photoautotrophic which enhances growth; the enhanced oxygen and carbon dioxide availability in the temporary immersion system allows aerobic respiration and photosynthesis to occur without the build-up of ethylene in the vessels. The exchange of gases in the RITA® system could be one of the factors leading to the increased growth rates observed.

This study indicated that plant quality is important for 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 (Figure 7). 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. The concentrations of $O_2$ in the vessels affect the root system and where an anaerobic or low $O_2$ availability 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.

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 3). In this regard, percent rooting and acclimatization 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*: 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 US $

| Materials or activity | Semi-solid (6 months) | RITA (3 months) |
|---|---|---|
| Media | 616 | 104 |
| Transfer | 518 | 155 |
| Media preparation (labour) | 518 | 104 |
| Autoclaving | 21 | 7 |
| Washing | 518 | 26 |
| TOTAL | 2 191 | 396 |
| Cost of vessels | 649 | 5 909 |
| FINAL TOTAL | 2 840 | 6 305 |

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 and 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.

A cost analysis was done (Table 4) using the average yields for all the clones. 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. 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 placed, with care, so that each stem is at a suitable 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. 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. This increased multiplication in the RITA® system was achieved in a smaller production space compared with that of the semi-solid system. Approximately 1,792 and 3,200 plants per $m^2$ at the onset of multiplication, for the semi-solid and RITA® systems respectively. 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 initial outlay for the RITA® vessels is high, but the vessels are re-used and this high cost is soon offset by the multiplication rates and turnover of the plants produced. The average yields (cold-tolerant together with sub-tropical) obtained from the RITA® system are greater 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. 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 large 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.

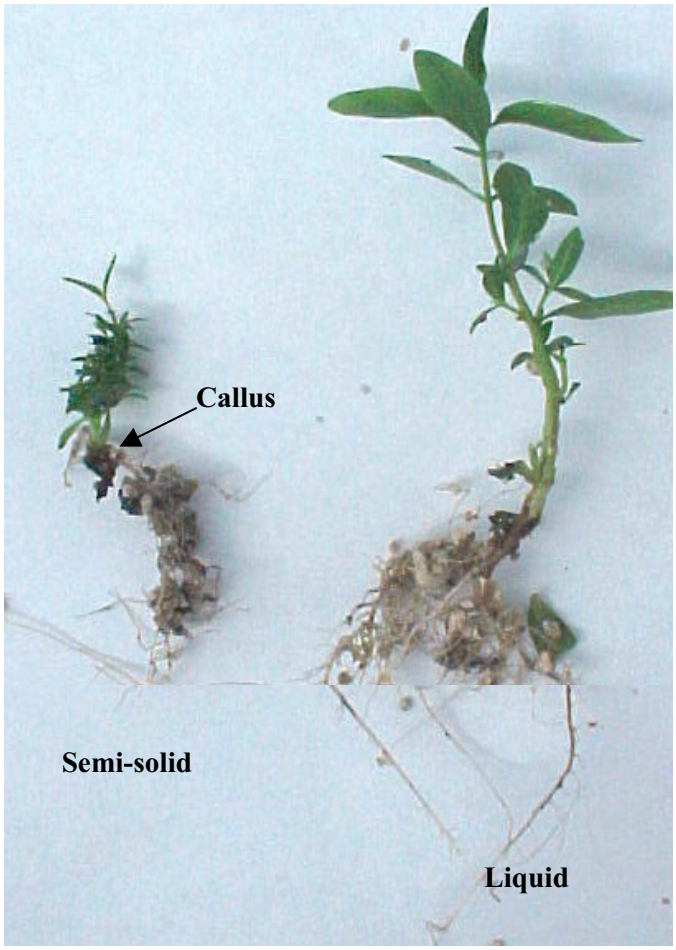


*Figure 7:* Rooted plants grown on semisolid medium and in RITA® vessels (right) after 4 days in the greenhouse.

## 4. Conclusion

The results indicate that for *in vitro* culture of *Eucalyptus*, the RITA® system results in benefits not yet 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 microbial-free plants. In RITA®, multiplication increases with the use of the appropriate numbers of starting

shoots in the vessels, as well as with appropriate exposure to media at correct intervals. Plant quality (hardiness and size) for clones tested to date is better in RITA®-produced plants than for plants grown in semi-solid media. In addition, cold-tolerant *Eucalyptus* clones which have extremely difficult to multiply, root, and subsequently acclimatize using semi-solid protocols, have been shown to respond favourably to the RITA® environment. Costs per 10,000 plants produced using RITA® 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.

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