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Current techniques and prospects for the clonal propagation of hardwoods with emphasis on *Eucalyptus*

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Abstract

Mass vegetative propagation has become an important tool for increasing the competitiveness of the forestry based industry. This method reaches its highest potential when it is used to establish clonal forests of hybrids endowed with better wood quality and higher volumetric growth. However, in several hardwood species, most notably in Eucalyptus, the popular method of rooting stem-cuttings has limitations, for example, rapid loss of rooting competence due to ontogenetic aging, intra-clonal variation resulting from topophysis, and poor quality of the root system, that in combination negatively affect genetic expression of some clones.

Two alternative super-intensive systems, one micro- and one mini-cutting, for cloning Eucalyptus

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at a commercial scale have been applied successfully. These systems have shown great potential for offering technical and economic advantages not available from conventional stem cuttings. The micro-cutting system uses the apices obtained from micro-propagated plantlets, while the mini-cutting system is based on the rooting of axillary shoots from rooted stem-cuttings. In both systems the plants are managed intensively to produce small cuttings. Field clonal hedges are replaced by indoor hydroponic mini-hedges, which provide plantlets or rooted cuttings with a high degree of juvenility. The success of the system is also dependent on achieving optimal nutrient status in the resulting mini-cuttings.

Compared to stem-cuttings, the micro- or mini-cuttings systems have improved rooting potential, rooting speed, and root system quality, as well as reduced costs. Additionally, these systems offer propagules with increased uniformity, and greatly reduced topophysis effects. The development of these super-intensive cloning systems has set the stage for a new phase of mass vegetative propagation of Eucalyptus and other hardwood species.

Another important tool for mass propagation of hardwoods at high multiplication rates is in vitro somatic embryogenesis, a technique whose detailed understanding can benefit from new methods of gene expression analysis. Better regeneration rates are required for the commercial viability of most hardwood somatic embryogenesis current procedures. As gene delivery and transformation protocols for tree species improve, embryogenesis methods will grow in importance, since they create suitable target tissues for gene transfer.

Nonetheless, at the core of every clonal propagation technique, lies the process of adventitious rooting. In spite of concerted efforts in the last three decades to fully understand the mechanisms of this developmental process, relatively little is known of the exact underlying steps that lead to the formation of a new root system in cuttings. Significant progress has derived from detailed physiological studies, the analysis of rooting mutants, and Agrobacterium rhizogenes-induced rooting. More recently, the global analysis of rooting-associated gene expression using DNA arrays has proven a very promising methodology to analyze complex developmental events. It is expected that new commercial clonal propagation protocols for hardwoods will emerge from some of these basic studies, as the understanding of the genetic control of adventitious rooting improves.

Introduction

Since the first development of cloning, and until its recognition as an operational method of reproducing superior trees, cloning of *Eucalyptus* species has undergone a continuous process of improvement through incorporation of new technical concepts and technologies at different phases of the process (1,2,3,4).

In the last two decades, cloning of *Eucalyptus* spp. has produced substantial progress for forest companies, especially for solving problems associated with diseases such as canker (*Cryphonectria cubensis*) and for improvements in productivity (1,3). Also, the focus of cloning has shifted to industrial requirements, rather than remaining limited to disease resistance and volume increase. The wood properties that positively influence industrial processes and product quality are considered, especially where cloning has an important role to play.

In the era of global markets, the development of forestry plantations for industrial purposes must aim for, among other objectives, increasing industrial competitiveness in the distinct market segments they interact with. In such a scenario, forestry based companies must consider the mode in which the forestry raw material can affect their competitive capacity. The modern concept of competitiveness includes producing products to meet the customer's requirements at low costs, in a sustainable manner and with minimum impact on the environment. Therefore, developing tree breeding programs to obtain quick gains, and also developing cloning systems to have a well established vegetative propagation method becomes important. The vegetative propagation methods should rapidly transform genetic gains obtained through breeding or genetic transformation, into benefits for the industry. One of the most efficient tools to acquire these objectives is the combination of inter-specific hybridization and establishment of clonal forestry derived from superior hybrid individuals (5).

In this context, hybridization is an alternative promising great impact at relatively low cost in tree breeding programs, and which can combine superior wood characteristics with tolerance to biotic and abiotic stress, thus providing a source of superior individuals, capable of yielding genetic gains in forest productivity and wood properties. Crossing species of different characteristics allows production of complementary wood properties in trees particularly in order to meet industrial requirements.

Considering that *Eucalyptus* hybrids are heterogeneous, the effective and quick integration of genetic gains obtained with hybrids into the industrial process depends basically on the existence of functional large scale cloning systems. Mass vegetative propagation perfectly complements hybridization for producing clonal forestry, and has some advantages over the sexual methods of mass reproduction of selected families, besides being the best way to commercially exploit the heterosis found in several *Eucalyptus* hybrid crosses. By capturing the total genetic variance (6), vegetative propagation allows for maximum benefits of wood properties and productivity, as well as production of more uniform raw material, which is highly beneficial to industrial processes and product quality. Therefore, the tree breeding programs that focus on these aspects will have great impact on the three important components of the competitive process: productivity, product quality and production costs.

Cloning systems have been based on the rooting of cuttings obtained from superior hybrid genotypes. In recent years, there has been a trend towards a shift from *in vitro* based-methods and the standard rooting of stem cuttings to super-intensive greenhouse systems (mini-cutting). In short, current methods are based on the continued harvest of propagules in a state of greater juvenility than can be achieved using rooted cuttings. The subsequent maintenance and extension of juvenility is managed by controlling environmental conditions, especially mineral nutrition, photoperiod and light quantity. The physiological mechanisms underlying such responses and the rapidity with which adventitious roots develop are not fully clarified, although progress has been made with *in vitro* methods, under highly controlled conditions. Another important mass clonal propagation technique is somatic embryogenesis, which corresponds to the formation of an embryo from non-gametic cells. It allows high multiplication rates, is amenable to scale up, artificial seeds allow the possibility of temporary storage of embryoids and, most importantly, embryogenic cultures are good targets for gene delivery protocols.

The improvement of total gene expression analysis by DNA microarrays and of localized gene-expression techniques by *in situ* hybridization, along with the detailed analysis of genotypes and/or mutants with distinct adventitious rooting capacity and of adventitious root induction by the soil microbe *Agrobacterium rhizogenes*, can shed further light on the control of adventitious rooting. Also of extreme importance are the recent efforts aiming at the sequencing of functional genomes of hardwoods, *e.g. Eucalyptus*, which will largely facilitate the analysis of sequential gene expression associated with critical developmental events, such as adventitious rooting. On the path towards that target, one may expect the development of new methods that can be added to the current best practice mass clonal techniques.

In its first part, this chapter basically describes the recent evolution of cloning of *Eucalyptus* as a model hardwood for industrial purposes in Brazil, with emphasis on the cloning techniques, exogenous factors controlling adventitious rooting and on the systems used for mass production of plant propagules. In the second part, the chapter briefly outlines the current status of somatic embryogenesis techniques in hardwoods and discusses recent advances in the understanding of endogenous factors controlling adventitious rooting, as well as the potential application of some of this knowledge in industrial mass cloning of hardwoods.

Cloning techniques

Tree species subject to early development of clonal propagation, particularly in temperate climates, include poplars, willows and aspens. One of the main features of poplars and willows is their excellent ability to propagate vegetatively by rooting of stem cuttings. This has been done both in nursery and field settings. Briefly, the production of *Salix* cuttings has been done from axillary shoots developed upon removal of the shoot apex. These axillary shoots can be harvested repeatedly for relatively long periods. Cuttings may be used as stock plants for various propagation cycles, resulting in improved rates of propagation. Similar procedures have been successfully applied to aspen and cotton-type *Populus* for the establishment of research plantations (7). The rooting of some recalcitrant species of poplars, aspens and willows may be improved by crossing with easy-to-root species. Some *Populus* species may need to be cultured *in vitro* to solve rooting problems. *In vitro* propagation of angiosperms has been done mainly by axillary budding; cultures in rich nutrient medium supplemented with appropriate amounts of auxins and cytokinins have even been able to rejuvenate mature tree clones, which may reach rooting rates comparable to those of juvenile clones (8). Poplar species of the *Leuce* section may be propagated by root segments. When planted in nursery beds, dormant root segments originate new plants, similar to sprouts formed from roots in the field upon cutting and burning of natural stands. This technique can be combined with tissue culture methods to propagate hybrid aspen clones (7).

Considering the consolidation of cloning as a tool for the establishment of productive *Eucalyptus* clonal forestry and its positive effects on industrial processes and product quality, the evolution of the systems used for cloning *Eucalyptus* was certainly expected. The precursor of this development started in the early 80's when cloning *Eucalyptus* by rooting stem-cuttings reached an industrial dimension (1). Since that time, the major constraint for general adoption was its applicability only to a small number of species and clones. Rooting of stem-cuttings was not suitable for a large number of

economically important species, including those important for energy and charcoal, like *E. citriodora, E. maculata, E. paniculata* and *E. cloeziana*, and an important number of clones of rootable species had sufficient problems as not to be available for commercial propagation. Most of the problems were associated with accelerated maturation processes causing rapid loss of rooting-predisposition, and manifestation of topophysis effects. The phenomenon of topophysis affects clones in different intensities, and is the main cause of intra-clone differences in growth and reductions in rooting ability. Franclet et al. (9) emphatically pointed out that topophysis induces physiological differences, and these differences can result in intra-clonal variability that can nullify the potential advantages of cloning. Another limitation of stem cuttings was associated with alterations of root system architecture, leading to root deformation. For many clones such deformations prevented their full genetic expression, consequently reducing the ratio between selected trees and number of clones effectively available for plantation use. Because of such limitations of rooting stem-cuttings, alternative methods were developed for commercial cloning of *Eucalyptus* species.

Micro-cuttings

Based on the work of Assis et al. (10), this technique was developed in Brazil in the early 90's. These authors observed that rooting ability of stem-cuttings decreases with ontogenetic aging and that the decline may be faster than previously reported in the literature. In *E. grandis* for example, rooting competence decreased from the fourteenth node up (11), while it was further delayed in *E. deglupta*. Assis et al. (10) observed that clones of *E. saligna, E. grandis* and *E. urophylla* that had equally high proportions of rooting *in vitro*, showed different intensities of decline in stem-cutting rooting percentages when managed in clonal hedges. This indicated that some factor related to clone growth, encompassing the period between planting and cutting harvest (6 months), could be responsible for these differences. Preliminary tests done at Aracruz Guaíba (unpublished) showed that, independent of species, almost 100% of the very young mini-cuttings obtained from the cotyledonary leaf pair rooted and the same tendency was maintained in the difficult to root species like *E. citriodora, E. cloeziana, E. paniculata, E. dunnii*, and *E. globulus*. However, with age, ranging from a few days to months, the cuttings harvested from such young plants showed a marked reduction in their rooting ability and in some cases such ability was totally lost. These observations suggested that the rooting potential reaches the maximum value at a high level of juvenility (mini-cuttings from cotyledons) and is similar in all species tested. But the decrease in rooting ability with seedling age differed among species, and this was a similar result to that found in the older materials in the field. This suggests that, at some stage, part of the juvenility obtained through the rejuvenation process *in vitro* (12) and/or on basal sprouts of cut adult trees (13) becomes gradually eroded during the growth of the clones in clonal hedges.

Thus, hypothetically, the rooting ability of *Eucalyptus* clones *ex vitro* should increase if the "physiological distance" between the maximum juvenility stage, obtained *in vitro*, and the propagule collecting stage is reduced. To test this hypothesis Assis et al (10) used shoot apex (micro-cuttings) of very juvenile micro-propagated plants of an *E. saligna* clone as propagules. These micro-cuttings had 30% higher rooting than the

stem-cuttings. In order to verify the results in a more representative sample, the rooting rates obtained by the traditional and the new micro-cutting method in seven clones of *E. saligna* and five clones of *E. grandis* were compared. Considering all the clones, rooting of micro-cuttings, was, on average, 18% higher than rooting of stem-cuttings, which amounted to an increase from 6.3 to 44.6 percent points above average. In general, larger increases in rooting percentage were observed in clones with lower rooting ability as stem-cuttings.

Clone micropropagation was carried out using sprouts obtained from just-rooted rejuvenated cuttings. Explants were washed for 20 min under running tap water and sterilized by successive immersion in neutral detergent 1.0 ml/l + alcohol 70% (v/v) (1 minute), Tecto 600® 1.5 g/l (2 minutes) + active chlorine 0.3% with a drop of Tween 20 (2 minutes), followed by rinsing with sterile water. The initiating medium consisted of Murashige and Skoog (MS) salts and vitamins with NAA (0.01 mg/l) and BAP (0,1 mg/l) in agar at 6.0% (w/v). In the multiplication phase NAA and BAP had their concentrations changed to 0.1 mg/l and 0.08 mg/l, respectively. The cultures were sub-cultured every 3-4 weeks and grew in a controlled environment at a temperature of 25 ºC and 1200 lux of luminous intensity. The elongated shoots were rooted in a medium containing KNOP macronutrients, Fe EDTA and micronutrients under the same environmental conditions.

Other results were obtained from several other trials established to define substrate, growth substances, environmental conditions for rooting, etc. One of the most significant findings of this new technology was the complete elimination of the use of growth substances usually required for the rooting of stem-cuttings (10). These substances did not increase rooting of micro-cuttings, but in some cases reduced it, indicating that the endogenous auxin concentrations in the juvenile tissues were sufficient to promote rooting. Based on these results, a super-intensive system of *Eucalyptus* propagation *ex vitro* was established.

The main feature of the technique is the use of juvenile plants, or plants rejuvenated in vitro, as sources of vegetative propagules. Shoot apices are used as micro-cuttings, which are placed for rooting in a glasshouse equipped with temperature and humidity controls. The actual size of micro-cuttings is about 7 to 8 cm with two to three leaf-pairs. Presence of the shoot apex is important for achieving a good quality root system, because its presence induces a taproot-like system. The micro-stumps left after micro-cutting harvest, sprout rapidly, producing new micro-propagules which can be harvested for use within a period of 15 days in the summer and 30 days in the winter.

Since its first use, the micro-cutting technique has been improved continuously by incorporating new research findings. The evidence of the evolution of this technique is well-documented in publications of Assis et al. (10); Xavier & Comerio (14); Iannelli et al. (15); Assis (16); Wendling et al. (17); Higashi et al. (18), and Campinhos et al. (19). The participation of a number of dedicated scientists was of fundamental importance for the establishment of an efficient, super-intensive cloning system for *Eucalyptus* species. The various technical contributions were exchanged in a pre-competitive development model, with intensive information exchange, and were the basis for the rapid evolution of this new concept of cloning *Eucalyptus* and other hardwoods on a large scale.

Figure 1. Sequence of the micro-cutting technique - Micropropagation (A); rooted micro-cuttings (B); mini-clonal hedge (C); large scale micro-cutting rooting (D).

Mini-cuttings

Probably the first initiative to root mini-cuttings in *Eucalyptus* was taken in the early 80's, by using shoots obtained from thinning operations with rooted stem-cuttings (unpublished). The early trial results were discouraging, due to inconsistent results that varied from highly positive to negative. Later studies showed that such inconsistencies were caused by nutritional deficiencies in the mother plants at the time of mini-cutting collection. A similar system called “cascade propagation”, used in France, is described by Chaperon (20). However, it was only in the 90's, after the consolidation of the micro-cutting as a functional propagation system, that the mini-cutting system became commercially viable for *Eucalyptus* cloning. Higashi et al., (18) developed a functional and efficient mini-cutting cloning system and several other researchers added significant contributions to the development of this technique (17,19,21).

Mini- and micro-cutting techniques are very similar in both concept and operational procedures, differing mainly in the origin of the initial propagules. The micro-cuttings are obtained from shoot apices originating from micro-propagated plants, and the mini-cuttings come from axillary sprouts of plants cloned as stem-cuttings. After rooting of the first shoot, the two techniques are identical. In some clones, the use of mini-cuttings requires a sequence of propagation cycles (serial propagation) to obtain the necessary reactivation for acquiring their full potential for rooting capacity. In micro-cuttings, such propagation cycles come naturally through monthly *in vitro* sub-culturing of explants. Micro-propagation is unnecessary for easy-to-root species because high levels of juvenility can be obtained easily by inducing basal shoots; therefore, in such cases, the mini-cutting approach is both technically and economically feasible.

Despite the fact that success of these techniques is believed to be related to the maintenance of a very juvenile stage (10,14), new findings suggest that their high

rooting potentials are also related to the better nutritional status of the mini-cuttings. In general, mini-cuttings or micro-cuttings root better than juvenile stem-cuttings of the same clones produced in clonal hedges in the field. Both kinds of material are juvenile, but the mini-and micro-cuttings cultivated in well-balanced, nutritive solutions root better, as a result of improved nutritional status. The rooting superiority of mini- or micro-cuttings may also be related to a differential amount of lignification in the two groups of propagules. Compared to stem cuttings, these micro- or mini-cuttings can be considered as "herbaceous", and in using these techniques many complications associated with lignin formation and its increased concentration in tissues can be avoided.

Although the two techniques are very similar, they have received different names. The terms mini-cutting and micro-cutting were designated, by convention, to systems of vegetative propagation that have their origin in rooted cuttings and micro-propagated plants, respectively. Nevertheless, there is a tendency to change this terminology to avoid confusion, with the traditional term 'micro-cutting' reserved for rooting of shoots produced *in vitro*. Therefore a system of vegetative propagation *ex vitro* based on mini-propagules could be termed as 'mini-cutting', independent of its origin, including those originating from a micro-propagation system. Henceforth, we will use the term mini-cutting to designate both techniques.

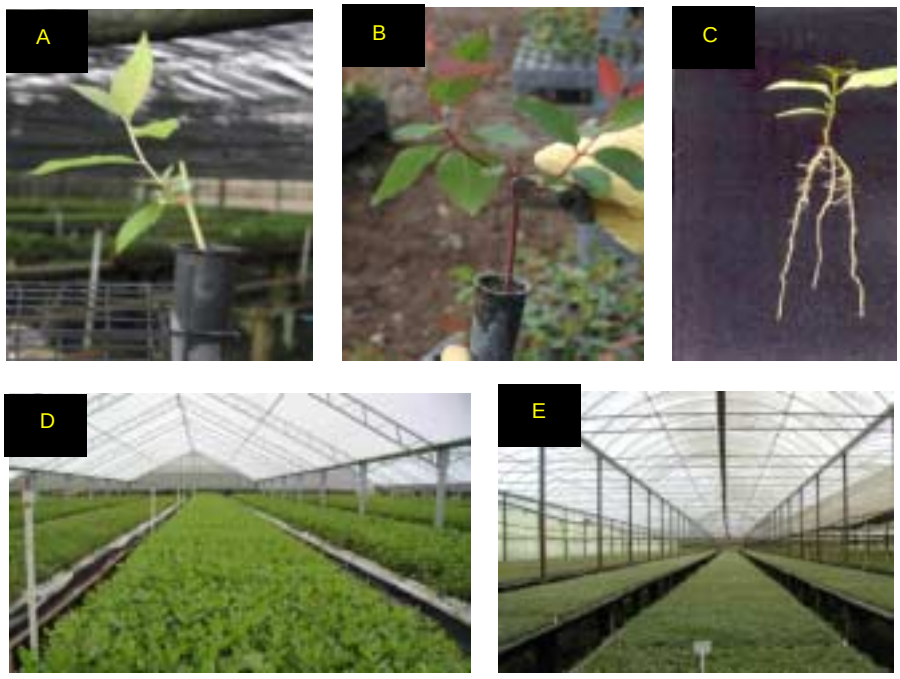

Figure 2. Sequence of mini-cutting technique- Rooted macro-cutting (A); collecting sprouts from macro-cutting (B); rooted mini-cutting (C); mini clonal hedge (D); large scale rooting mini-cuttings (E).

Advantages of mini-cuttings

Compared to the traditional rooting of stem-cuttings, mini-cuttings have many advantages, leading to operational, technical, economic, environmental and quality benefits. Operationally, the labor and cost demands are markedly reduced, due to elimination of labor intensive treatment with growth substances, and many other operations required in an extensive outdoor clonal hedge management system. Many field operations like soil preparation, fertilization, irrigation, cultivation, weeding, pest and disease control, sprout transport, etc are replaced by intensive activities in smaller indoor areas at much lower costs, where the amount of chemicals used is also drastically reduced.

The rooting ability of mini-cuttings is much higher than for the stem-cuttings. Although the benefits vary among the species and clones, in general those species or clones that are 'poor rooting' with stem-cuttings perform better with mini-cuttings, which may have increased rooting in the order of 40%. The main reasons for such increases are related to both higher levels of juvenility and optimal nutritional content of the tissues, which improves the rooting predisposition and speed of root initiation. The rooting speed of mini-cuttings has two other important consequences in a commercial cloning program: the time that the plants are held indoor is usually reduced to half compared to rooting of stem-cuttings, thus considerably improving use of facilities and reducing the exposure of basal tissue of mini-cuttings to pathogenic fungi. At the initial and more susceptible stage, the faster reaction of mini-cuttings in rapidly inducing wound callus formation at the basal end provides protection against pathogens, resulting in reduced fungicide applications.

Mini-cuttings produce better quality root systems with a tendency for development of a taproot-like system, in contrast to the predominant lateral root growth habit in the stem-cutting system. Apparently the connection between root and stem tissues in the mini-cuttings is more suitable due to lower lignification of the tissues involved. This kind of root system appears to be more responsive to fertilization, is more resistant to environmental factors, and considerably reduces intra-clone variation. As pointed out earlier, the topophysis effects observed in several systems of vegetative propagation are highly harmful to clonal uniformity. In this respect mini-cuttings offer the advantage of physiological homogenization of propagules, producing a much more uniform forest.

Evolution of mass production of vegetative propagules

When the rooting of stem-cuttings was first introduced commercially in Brazil (1), the vegetative propagules for establishing clonal forests were obtained from field plantations. This management strategy required annually reserving of large areas of plantations, with the only objective being that of obtaining cuttings for commercial rooting programs. Although the commercial plantations could be used to produce propagules, the high time-demand to produce both wood and vegetative propagules made this alternative unfeasible. The clonal hedge-based concept of intensive management was introduced sometime later. This system demanded smaller areas due to high shoot productivity per unit area (18,22). Despite being a tremendous advance for achieving mass production of shoots, clonal hedges were still complicated to manage and poorly controlled. To meet the demands of clonal forestry, the system still required

large areas, intensive labor, and large inputs of nutrients and water. The propagators were strongly dependent on the weather since the system was completely subject to environmental conditions.

Figure 3. Evolution of mass production of vegetative propagules in *Eucalyptus*. Clonal bank (A); clonal hedge (B); indoor hydroponics mini clonal hedge in sand bed (C); indoor hydroponics mini clonal hedge using intermittent flooding.

Mini clonal hedges

At the beginning of the last decade, the development of micro-cutting technology for *Eucalyptus* (10) allowed the concept of super-intensive management of producing vegetative propagules to be achieved at a commercial scale. As for cloning methods, the advent of the micro-cutting system substantially contributed to the progress made in systems for large scale production of vegetative propagules *ex vitro*. Originally the system was based on mini-hedges established through rooted mini-cuttings, grown in small containers (dibble tubes). This system provided a series of technical and economic benefits as well as good root quality (10,14,16). Despite representing a great advance over the clonal hedges in the field, mini-hedges faced some limitations. The outdoor mini-hedges were still hostages of climate, and problems related to adequate maintenance of nutritional status and leaf diseases continued, especially during winter. The main problems were: reduced photosynthesis rate, reduced nutrient uptake and high levels of nutrient loss by leaching during periods of excessive rainfall, or even during irrigation. These limitations led to the development of an indoor mini hedge system.

Indoor hydroponics clonal hedges

The development of new technologies contributed to an increase in the efficiency of the existing systems. Hydroponics concepts for production of mini-cuttings were introduced first in an operational indoor system based on drip fertigated sand beds (18). The major contribution of this system was in terms of assuring an adequate nutritional status of the mini-cuttings, which is the key factor to obtaining high rooting percentages in juvenile vegetative material. Using the same concept, Campinhos et al. (19) developed a highly efficient method based on an intermittent flooding system, where the containers of the mini-stumps are immersed in a nutritive solution for fertigation. These two hydroponics methods, sand-bed and intermittent flooding, are most widely used and most Brazilian companies use one or other of them.

Virtual clonal hedge

The observation that the shoots from a first collection root better than those after the third or fourth collection indicated that vegetative propagules could be produced anywhere, without allocation of a definite physical area. Assis (16) proposed a system in which mini-cuttings can be produced in virtual clonal hedges. Any rooted plant with normal growth, when it reaches a height of about 15cm, has its apex (7 to 8 cm) cut back to produce a new mini-cutting. As soon as the newly rooted mini-cuttings are about 15 cm high, their apices can be used as a new source of mini-cuttings, thus creating a virtual mini-hedge after some cycles of re-rooting, and requiring no specific area for production of vegetative propagules. The important attribute of this system is that the plants used for producing mini-cuttings can be planted in the field without losing the quality achieved from the intermittently flooded mini hedges.

The sprouting of harvested plants becomes an extra option for providing mini-cuttings. The blanks resulting from rooting failure can be filled with mini-cuttings produced on the mini-stumps of the harvested plants. Similarly, the sprouts produced on both the pruned plants and the apices of the rooted mini-cuttings can be used continuously in a self-sustained cyclic process. This system represents an excellent technical, economic, and operational alternative, but it only works well if the mini-cuttings are supplied with good nutritional conditions. An extension of sub-fertigation to the treatment of newly rooted mini-cuttings is the most suitable option to make virtual mini-hedging practical. The use of very soft mini-cuttings must be avoided in order to prevent fungal infection. Locations subject to wide climatic variations and without any environmental control are less suitable for establishing virtual mini-hedge systems, therefore they are not recommended in situations where environmental control is not feasible.

Advantages of indoor hydroponic mini hedging

The development of the concept of super-intensive, hydroponic mini hedging brought several benefits to commercial cloning of *Eucalyptus*. All indoor mini hedging systems have technical and economic advantages relative to normal outdoor clonal hedging. Higher productivity of mini-cuttings, lower labor demand and low consumption of chemicals and water, represent major sources of economic gain. In addition, considering the important effect on rooting success of the conditions under which mother-plants grow (23,24,25,26), the main technical advantages are related to these

same aspects. These systems allow the implementation of important theoretical concepts, which are difficult to implement outdoors, such as CO_2 enrichment, and control of temperature, light intensity and photoperiod. These factors, in addition to nutrition, are of fundamental importance in enhancing rooting predisposition. This system also enables application of pre-harvest treatments, such as spraying mother plants with growth substances (phytohormones) to increase rooting potential.

Propagule productivity

Eucalyptus cloning on a large scale started in Brazil using very extensive systems, and the productivity of vegetative propagules was reported as averaging 114 cuttings/m^2 of field hedges (1). With the introduction of the mini-hedges production increased progressively to 121 cuttings/m^2 (22), then to 1,752 cuttings/m^2 per year (18), and presently is at about 24,000 cuttings/m^2/year. Table 1 provides an overview of the evolution of the productivity of vegetative propagules in *Eucalyptus*. The super-intensive systems, especially those that are based on hydroponics, are much more efficient, having a 350-fold higher productivity than the initial systems used in Brazil. Comparing these different systems, the substantially greater production capacity for producing vegetative propagules in the super-intensive systems means that more cuttings can be produced per year and per unit area of mini hedges. In addition, they are easy to handle and manage, and their production requires a lower cost per plant.

Variations in the productivity of mini-cuttings as observed in Table 1 resulted from different hedging systems, from use of different genetic materials, and also from application of different management practices. Clones of *E. grandis* x *E. urophylla* hybrids are generally much more productive as mini-cuttings than are those of other species. The majority of the pure *E. saligna* clones are much more difficult to produce as mini-cuttings than the *E. grandis* x *E. urophylla* hybrid, for which production/mini-stump is almost doubled. Even so, the rooting competence and propagule productivity is much higher than in the outdoor clonal hedges. The other source of variation in productivity is related to the kind of management adopted to utilize the sprouts.

Certain practices for managing mini-stumps in sand beds produce vegetative propagules similar to the macro-cutting system. The sprouts are allowed to grow to bigger sizes, and after collection each sprout is divided into three mini-cuttings (basal, middle and apical). This system produces higher number of propagules/m^2 but it loses the best characteristic of the mini-cuttings provided by the presence of the shoot apex, which is the production of a high quality root system (a taproot-like system, as discussed earlier).

In both the hydroponics systems described above (i.e. drip fertigation and intermittent flooding) losses of mother plants are common due to salinisation, excessive harvesting, diseases etc. In this respect, the systems that use containerized mother plants, as in intermittent flooding, have advantages over the sand bed systems. In sand beds, the replacement of dead plants, and the need to get them to a productive stage requires more time when compared to the intermittent flooding system.

The most attractive characteristic of virtual mini-hedges, i.e. which do not require allocated physical areas for producing vegetative propagules, is that the mother plants are themselves also plantable. After supplying mini-cuttings, such plants are managed as normal rooted mini-cuttings. In the intermittent flooding system, the mother plants also

can be planted, provided that the harvesting regime has not degraded their quality. Under successive and heavy harvesting regimes the quality of mother plants frequently declines.

One question that arises is the influence of plant spacing in mini-hedges on the rooting of mini-cuttings and mini-stump productivity. Trials at Aracruz Guaíba showed that 5 x 5 cm spacing between plants does not interfere significantly in the rooting capacity of the mini-cuttings, but wider spacing allows for higher survival rates of mini-stumps. The final results are better with narrower spacing, however, since there is a more than proportional compensation by the presence of a higher number of plants/m^2.

Table 1. Propagule productivity in different clonal hedging systems.*

System	Species/ Spacing (m)	Propagule Productivity (/m^2/year)	References
Clonal bank	*E. grandis x E. urophylla* 3.0 x 3.0	114	(1)
Clonal Hedging	*E. grandis x E. urophylla* 1.0 x 1.5	121	(22)
Clonal Hedging	*E. grandis x E. urophylla* 0.5 x 0.5	1752	(18)
Mini hedging Outdoor	*E. grandis x E. urophylla* 0.05 x 0.05	29200	(14)
Hydroponic mini hedging (sand bed/drop irrigation)	*E. grandis x E. urophylla* 0.1 x 0.1	41480**	(18)
Hydroponic mini hedging (intermittent flooding)	*E. grandis x E. urophylla* 0.05 x 0.05	25500	(19)
Hydroponic mini hedging (intermittent flooding)	*E. grandis x E. urophylla* 0.05 x 0.05	24000	Aracruz Guaíba operational
Hydroponic mini hedging (intermittent flooding)	*E. saligna and E. urophylla x E. globulus* 0.05 x 0.05	14400	Aracruz Guaíba operational
Virtual mini hedging	*E. saligna and E. urophylla x E. globulus* 0.05 x 0.05	-	Aracruz Guaíba operational

* Based on cuttings/mini-cuttings/stump considering 12 months of effective production, without discounting the replacement of dead plants.
** Subdividing sprouts in 3 mini-cuttings.

Temperature and light

Temperature and light are good examples of factors that can be controlled in mini hedges, to improve rooting predisposition of mini-cuttings. Light can be managed as to photoperiod and intensity. Light intensity can strongly influence cutting productivity and rooting by reducing or increasing endogenous phenolic substances (27) that can act as rooting inhibitors or promoters depending upon the concentration in the tissues and the species involved (28). Light can also affect the concentration of endogenous cytokinins, which can function as rooting inhibitors (29). Other potential effects of light include changes in either nutrient or auxin uptake (since light can affect stomatal opening and transpiration), and induction of photochemical or metabolic degradation of auxins (30). Promptness to root in cuttings of *Eucalyptus globulus* grown *in vitro* without exogenous auxin was reduced by light, whereas *E. saligna* was unaffected (31). The photoperiod,

especially combined with temperature, can influence the rooting predisposition of shoots. Light can also influence rooting by controlling internal levels of carbohydrates. Etiolation of mother plants can affect rooting and root number, which, depending upon the species, may either increase or decrease (32). This variation appears to be linked to carbohydrate production (33) and accumulation and transport of auxins (32).

Temperature can influence rooting by interfering with nutrient uptake and metabolism, and its control can be adjusted for optimum production of cuttings (especially in sub-tropical climates). Assis (16) observed that rooting percentage of mini-cuttings decreases in cold winter months. Recent work at Aracruz Guaíba (unpublished) showed that this problem could be solved by supplying additional light (14h/1000 lux) and increasing environmental temperature of mother plants (>20 ^{o}C). Since the two factors were not separated, the effect of each individual factor could not be determined. However, considering that nutrient concentration is of prime importance for the rooting process, and that nutrient uptake depends on metabolic activity, it can be assumed that both factors contributed to reestablishment of normal rooting competence. In general, in areas of cold climate, rooting is limited to warmer months only; therefore, as discussed above, if light and temperature are controlled, the use of greenhouse space can be optimized by extending the rooting period for the entire year.

Nutrition

One of the most important advantages of hydroponic mini-hedges is the possibility of providing well-balanced nutrition to mini-cuttings. The effects of macro- and micronutrients on root initiation of cuttings on several plant species have been well documented in the literature (23,26,32,24,25). As discussed earlier, nutrition is the key factor affecting rooting predisposition, because of its involvement in determining the morphogenetic response of the plants. In the hydroponics mini-hedging system nutrients are supplied in ideal concentrations to promote rooting. Hydroponics, without the influence of rainfall and of overhead irrigation, allows better nutrition control of mother plants. The use of indoor hydroponics mini-hedges allows nutrient supply in a correct concentration, avoiding the nutritional imbalance commonly observed in outdoor clonal hedges; in addition, there is no leaching by rainfall and the response to nutrient application is rapid. According to Haissig (25), a feasible nutritional balance of mother plants can be linked both to the production of tryptophan, a precursor of IAA, and to storage substances.

Calcium plays an important role in the rooting process, mainly because it acts as a peroxidase activator (25). The root formation step of *in vitro* grown cuttings of *Populus tremula* x *P. tremuloides* hybrids was inhibited by calcium removal and inactivation with either chelates or a Ca^{2+} channel blocker (34). According to the practical experience of Aracruz Celulose, the percentage rooting of *Eucalyptus* mini-cuttings decreased considerably if the leaf calcium concentration dropped below 0.7%. This situation was observed especially in winter when, in the absence of heating, leaf calcium concentration was substantially lower than in warmer months. Calcium deficiency induces shoot apex necrosis in mini-cuttings, which can cause problems of rooting and mini-cutting development (35). This phenomenon was commonly observed on the initial, operational scale outdoor mini hedges.

In general, moderate nitrogen deficiency can improve rooting (25), but at high levels more energy is required for vegetative growth and especially for leaf expansion. Consequently, carbohydrates are not stored at suitable levels, thus reducing the C:N ratio. On the other hand, extreme nitrogen deficiency can reduce rooting since it is necessary for nucleic acid and protein synthesis. Based on the considerations of Hartman & Kester (24), and Haissig (25,36) a general model for leaf nutrient concentration can be established. The mother plant should be well nourished with macronutrients like phosphorus, potassium, calcium and magnesium and should be moderately deficient in nitrogen.

Root architecture can be potentially modulated by patches of high nitrate, which tend to cause branching and elongation of lateral roots in many plants, although the majority of examined species are herbaceous. Nitrate has been identified as a subterranean signaling molecule controlling the branching pattern of roots, independent of its effects on nitrogen metabolism (37). This has been demonstrated by the fact that *Arabidopsis* mutants deficient in nitrate reductase still display root branching in nitrate rich patches; additionally, the identification of *ANR1*, a MADS-box transcription factor required in the nitrate induced root branching response, points to existence of a nitrate signal transduction pathway. Potential overlap of nitrate and auxin signal transduction pathways in the control of root architecture has been indicated by the observation that *axr4*, an auxin resistant mutant of *Arabidopsis* with defective root gravitropism and lateral root initiation, also lacks the nitrate induced branching response (38).

The effects of micro-nutrients on rooting are variable. Manganese, due to its inhibitory effect on rooting (possibly related to the promotion of auxin oxidative enzymes) should be used at minimal concentrations, but boron and zinc are essential for rooting (26). Zinc increases the endogenous content of auxins presumably by increasing tryptophan pools. A requirement for boron as a cross-linking agent between dimers of the pectic polysaccharide rhamnogalacturonan II in the primary cell wall has been shown by the isolation of growth- impaired *Arabidopsis* mutants that display 50 % less borate cross-linked polysaccharide (39). Rooting of micropropagated *Eucalyptus globulus* cuttings was improved by 10 % with the removal of boron from the root formation culture medium (40). Table 2 shows nutrient concentrations in leaves of well nourished plants (Roberto Ferreira Novais, pers. comm.) for rooting mini-cuttings. Monitoring leaf nutritional concentration of the mother plants, as a guide to ensuring adequate levels of macro and micronutrients has been an important factor in improving the rooting competence of mini-cuttings.

Phytosanitary advantages

Shoot contamination by splashing of soil during rainfall or over-head irrigation (3) is a very well known sanitary problem associated with outdoor clonal hedges. As many pathogenic fungi are soil-borne, the cuttings produced either in clonal banks or in outdoor clonal hedges, are frequently contaminated by these fungi. Thus, one of the advantages of using indoor hydroponics is better phytosanitary control. In the absence of leaf wetness, occurrence of leaf diseases is greatly reduced, thus producing pathogen-free cuttings with increased rooting yields. However, all these benefits of the indoor system can either be lost or become disadvantageous in the absence of efficient

controls to minimize the effects of external factors such as extreme temperature and humidity.

Table 2. Concentrations of macro- and micro-nutrients in leaves of well nourished *Eucalyptus* plants.

MACRO NUTRIENTS (%)						MICRO NUTRIENTS (mg/kg)				
N	P	K	Ca	Mg	S	B	Mn	Zn	Fe	Cu
2.5 to 3.0	0.2 to 0.4	1.5 to 2.0	1.0 to 1.5	0.25 to 0.40	0.15 to 0.25	40 to 70	100 to 500	50 to 60	100 to 200	10 to 15

Management of mini-stumps in hydroponics systems

The management of mini-stumps is relatively simple, but some aspects must be considered for the technique to be successful. Mini-cutting collection should be done selectively to avoid mini-stump degradation. Desalination should be done at least once a week by generously irrigating the substrate to leach the surface-accumulated salt. In non-automated nutritive solution preparations, the entire solution should be changed every 15 days. In intermittent flooding better results are obtained when the electric conductivity is adjusted to 1.8 – 2.2 in the winter, and to 0.8 – 1.0 in summer. Preventing cut and abscised leaves from falling into the nutritive solution (41) prevents biotic and abiotic problems in managing mother plants.

Figure 4. Aspects of mini-cutting management - Selective collection (A); Salination of mini stumps (B).

Plant Growth Promoting Rhizobacteria and substrate rhizobacterization to improve rooting and disease control

The rhizosphere, the zone under the influence of the root system, is a specialized and dynamic microecosystem, owing to a diverse quantity of compounds deposited in high quantity by the plant roots (42). It favors growth of various forms of microorganisms that benefit from the constant supply of organic substrates and factors essential for growth. The 'rhizosphere effect' induces a microbial population of more than 100-fold when compared to that in the adjacent soil (43). Although the effect is non-specific, the

bacteria are abundant because of their rapid growth and their ability to utilize a large range of substances as carbon and nitrogen sources (44).

The interaction between rhizosphere microorganisms and roots can be beneficial, neutral or harmful to plants and can vary according to the soil conditions (44). The term rhizobacteria is used to designate root-colonizing bacteria. Beneficial rhizobacteria are found in the rhizosphere of diverse crops, and 2 to 5% of the isolates can have positive effects on plant growth (45). The term "Plant Growth Promoting Rhizobacteria" (PGPR) is used to designate a subgroup of beneficial rhizobacteria that induce greater plant growth and/or productivity (46). This definition does not require any implication of biological control as a determinant of growth stimulation.

Recent studies done at the Forest Pathology Laboratory of UFV (Patent PI0101400 and PCT/BR02/00013) in collaboration with the major eucalyptus producing Brazilian companies have shown that substrate infection with selected isolates of rhizobacteria can stimulate rooting of cuttings and mini-cuttings (Figure 1), which, depending upon the

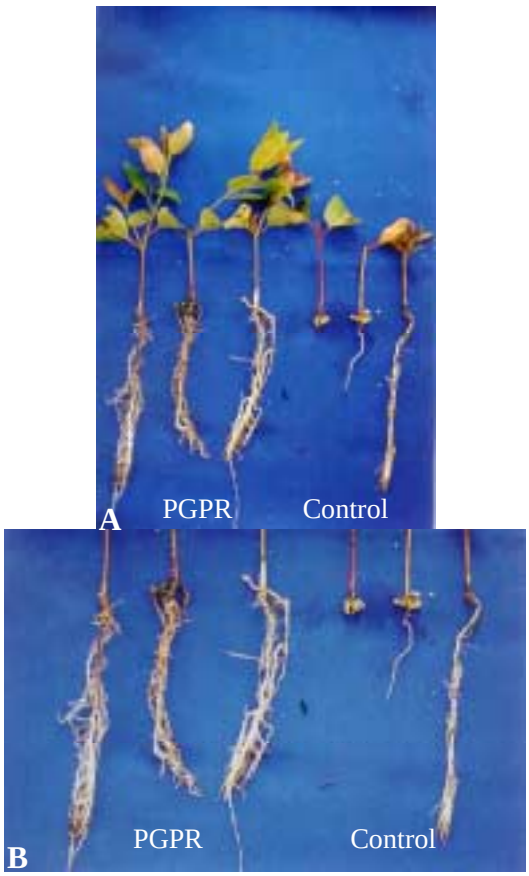

Figure 5. Effect of Plant Growth Promoting Rhizobacteria (PGPR) on *Eucalyptus* rooting (A); Close-up view of the root system (B); control plants are untreated.

clone and the isolate, can reach 100%. In addition, some of these isolates can induce control of rust (*Puccinia psidii*) and cutting rot (*Botrytis, Cyllindrocladium, Rhizoctonia* and *Sporothrix*).

Use of the mini-cutting system for cloning other woody species

The applicability of the mini-cutting technique has also been tested on other broad-leaf and coniferous woody species. In general, the methods used are similar to those used with *Eucalyptus*, with minor adaptations. The results have shown that this technique can be fully applied to species like *Pinus taeda, P. elliiottii, Acacia mearnsii* and *Ilex paraguariensis* ("mate") and many other woody and non-woody species. The best results were obtained with juvenile plants, as is the case with difficult to root *E. citriodora, E. maculata* and *E. paniculata*. Thus, the mini-cutting method is feasible for use in family multiplication in these species. Use of mini-cuttings can be a possible alternative for production of family forests by multiplication of full-sib or tested half-sib families.

Aracruz Guaíba started a small program of establishing *E. globulus* clonal plantations, based on adult and early selected trees resistant to rust caused by *Puccinia psidii* and other diseases. The preliminary results suggest that mini-cutting is a very promising technique to commercially propagate this species. Compared to stem-cuttings, mini-cuttings gave better results in both rooting proportion and root system quality. It appears that propagation of *E. globulus* is profitable, since it is considered to have different and higher nutritional requirements. The development of special nutritive solutions for *E. globulus* tends to improve the results and it is clearly possible to establish efficient clonal programs for this species.

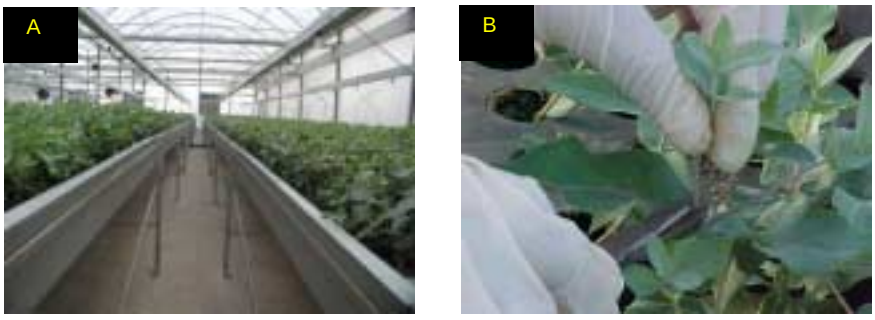

Figure 6. Mini-cuttings in *E. globulus* - Mini-hedge (A); mini-cutting collection (B); rooted mini-cutting acclimatization.

In *Acacia mearnsii*, the mini-cutting system has shown good results, despite this species being considered as recalcitrant with regard to vegetative propagation (47). Although reasonable success was obtained by Assis et al. (48) with adult trees, application of the mini-cutting system gave better results, indicating the possibility of developing clonal forestry in this genus. The key point in this case is to achieve juvenile or rejuvenated shoots to start the process. Induction of basal shoots (which do not

produce the tannin oxidation normally present in the wounded trees of this species) is necessary to establish initial healthy rejuvenated plants. Basal shoot induction should be carried out on standing trees to avoid problems with tannin.

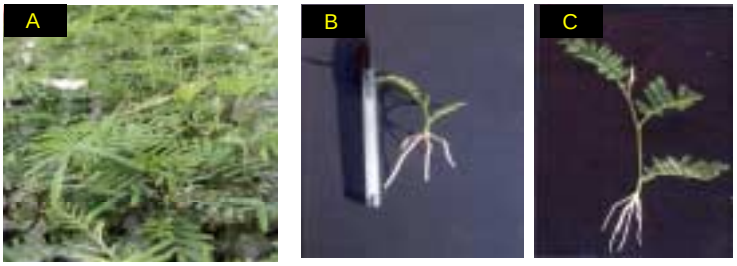

Figure 7. Mini-cuttings in *Acacia mearnsii* - Mini clonal hedge (A); rooted mini-cuttings (B and C)

In *Pinus*, only juvenile seedlings were tested as mother plants. *Pinus elliottii* seedlings can be easily rooted as mini-cuttings with more than 85% success. *Pinus taeda* also roots well although somewhat less (76%) than *P. elliottii*. These results suggest the possibility of developing family forestry programs with both species based on the systems used for *Eucalyptus*. Managing *Pinus* mini-hedges in hydroponics is efficient for providing mini-cuttings with a good nutritional status. The productivity per unit area of the pine mini-propagules, without any change in the nutrient solution, was 9600/m^2/year. The results of mini-cutting productivity and rooting proportion are, therefore, highly encouraging.

Figure 8. Mini-cuttings in *Pinus taeda* - Mini-cutting collection (A); large scale mini-cutting rooting (B); rooted mini-cuttings (C and D).

Somatic embryogenesis and cloning of hardwoods

The formation of embryos from somatic or haploid cells without gametic fusion is known as somatic embryogenesis. This *in vitro* technique is considered highly useful for large-scale propagation of elite plants. It is also useful for basic studies on embryogenesis, and for the production of transgenic plants. This last application is growing in importance, since embryogenic cultures are excellent targets for gene insertion. Somatic embryos (or embryoids) are very similar to zygotic embryos, presenting polarity and the same morphological developmental stages, but somatic embryos lack an endosperm and may display anomalous development (*e.g.* extra cotyledons or trumpet-like cotyledons). Somatic embryogenesis has been achieved for hundreds of species, some of which are hardwoods. In *Eucalyptus*, somatic embryogenesis has been reported for several species, including *E. citriodora* (49), *E. grandis* (50), *E. dunnii* (51), *E. nitens* and *E. globulus* (52) with various degrees of success. Nonetheless, in many cases, commercial viability of somatic embryogenesis protocols is made difficult by low regeneration frequencies in interesting clones, genetic variation in the starting material (often seed or seedling derived) (53), and high production costs.

Embryoids may arise from two distinct patterns: either directly from the explant tissue or indirectly from calli grown on the original explant. The first pattern results from cell re-differentiation, whereas the second is the result of dedifferentiation. Embryoids generally form from single cells. The development of embryoids is preceded by the establishment of dual polarity in embryogenic cells, *i.e.* the apical and basal pole from which shoot and root will originate, respectively. The underlying mechanisms controlling plant cell polarity are not fully understood, but they seem to involve the asymmetric distribution of calcium, auxin, secretory vesicles and F-actin, based on studies with fucoid algae and *Arabidopsis* (54,55). The basipetal flow of auxin in the plant axis established during embryo formation appears essential for the continuity of the vascular system in plant organs at later stages of development (55).

A general strategy for somatic embryogenesis in hardwoods involves two culture cycles: in the first, the explant is exposed to medium containing high concentrations of auxin and kept in the dark to induce formation of embryogenic cells; in the second, pro-embryos are encouraged to develop further by cultivating them in low auxin or auxin-free medium under light. Gene delivery protocols are normally applied prior to, or early in the stage of embryogenic cell induction, since transformed individual cells may give rise to non-chimeric transgenic plants. Several factors affect somatic embryogenesis, including the explant type (best results are normally obtained with young embryos, seedlings or ovules), nitrogen source and content (addition of reduced nitrogen is often advisable), carbohydrate content (generally sucrose at higher amounts in the first cycle), decreasing medium water potential (with mannitol or polyethylene glycol) or adding abscisic acid at later stages of embryo growth (often beneficial to embryo maturation) (56). In many aspects, somatic embryogenesis protocols try to reproduce physiological conditions known to occur in zygotic embryo development.

Alternative treatments for the promotion of somatic embryogenesis based on stress-related treatments of donor plants or explants have been reviewed (57). Transient exposure to *in vitro* starvation, chilling, non-optimal hormone concentrations and cell wounding have yielded improved embryo formation from sporophytic somatic cells in

some species. Exposure to starvation-stress medium, for example, has promoted somatic embryogenesis in *Quercus suber* (58).

The detailed understanding of the roles of each of the steps and treatments applied to tree species in order to achieve successful somatic embryogenesis will improve with the analysis of gene expression profiles associated with this developmental process in some representative species. For example, using two modern methods for gene expression analysis, gene differential display and DNA macroarrays, gene expression profiles during zygotic and somatic embryogenesis were examined in loblolly pine (59). Using such studies, culture conditions for somatic embryogenesis can then be adjusted to reproduce as closely as possible gene expression profiles associated with zygotic embryogenesis. More studies like this in hardwoods will be facilitated by the progress of ongoing functional genome sequencing programs of species such as *Populus* sp. and *Eucalyptus* sp.

Endogenous factors controlling adventitious rooting

Adventitious rhizogenesis in cuttings (ARC) is a unique and complex process. It involves associated stress responses such as wounding, changes in plant water relations, and loss of correlative influences due to separation from the original root system. ARC seems to be regulated by the interaction of multiple factors including phytohormones (auxins, cytokinins, ethylene and polyamines), carbohydrates, phenolic compounds, donor plant status (nutritional, water and light conditions), as well as genetic characteristics (60). Since the role of exogenous factors (*e.g.* temperature and light) in the control of ARC has been briefly discussed in the previous sections, the rest of the text will focus on the contribution of endogenous factors to the process.

Analyses of ARC in a number of woody species have led to the recognition of phases in the process with distinct features and requirements. Although the division of phases varies from author to author, a widely accepted proposal identifies three main physiological steps: 1) induction – defined as the period in which no morphological events are clearly observed, comprising the early molecular and biochemical steps preceding morphological modifications; 2) initiation – in which cell divisions take place, root meristems are formed and root primordia are established; 3) expression – corresponding to the period in which root growth and emergence from cuttings occurs (61). Obviously, since the formation of root primordia is generally non-synchronized, these steps overlap in time within a given cutting that is undergoing adventitious rooting.

The anatomical patterns of root formation can be divided into either direct or indirect paths (62). Some species can present both patterns depending on physiological conditions or developmental state. The direct root formation path assumes the existence of competent root forming cells which, upon induction, start dividing in polar fashion leading to root primordia formation. These primordia are frequently associated with vascular tissue. The indirect root formation path involves an initial non-competent state (i.e. cells are initially unable to respond to induction signals); after induction, non-directed cell divisions occur, often leading to the development of callus. Induced competent cells are then formed and, upon new induction (which can be an extended initial induction) these cells divide in polar fashion, establishing root primordia (62).

The later stages of root primordia development involve the establishment of vascular connections with the stem, and root emergence (63). In the direct pattern of root

formation, cells ahead of the induced vascular strand divide and differentiate into vascular tissue. In the indirect pattern of root formation (callus derived root), vascularization of the callus normally takes place in basipetal fashion prior to primordia differentiation. The final link is established from the primordium vascular tissue to the vascular strands in the callus. The new root grows through the stem tissues by crushing and/or by enzymatic dissolution of cell walls. Root emergence may be a limiting factor for rooting in some species. Potential barriers that can delay root emergence include fibers, sclereids and resin and secretory canals (63).

Analyses of biochemical changes during ARC, both before and during anatomical changes, were carried out in several species grown in a variety of conditions, and these allowed the identification of some endogenous factors regulating the process. Carbohydrates provide energy for biosynthetic processes necessary for rooting, such as nucleic acid biosynthesis, *de novo* enzyme formation, and cell division (64). There is substantial evidence indicating that best rooting is positively correlated with optimum total non-structural carbohydrate concentrations before and during rooting. Several enzymes increase their activities during certain phases of the rooting process, including respiratory enzymes and amylases (65). More specific roles for carbohydrates in ARC have not been identified mainly due to differences in experimental results. For instance, several studies using exogenous application of carbohydrates to cuttings are difficult to interpret due to lack of information on uptake, translocation and local osmotic effects.

Among the many factors involved in the determination of rooting capacity, a central role appears to be played by auxin activity, *i.e.* the net result of the regulation of auxin metabolism, and cell sensitivity to this phytohormone. Some of the widely confirmed evidence for a key role of auxins in rooting are: 1) auxins are the only chemicals that consistently enhance root formation in naturally responsive cuttings; 2) presence of buds and young leaves (auxin sources) often enhances rooting; 3) supplied auxins can generally substitute for natural sources of these phytohormones in relation to rooting; 4) endogenous auxin transport in intact stems is mainly basipetal, in agreement with root formation polarity; 5) auxin transport inhibitors can block root regeneration. Moreover, biochemical and genetic evidence for a fundamental role for auxins in adventitious root formation has resulted from the identification of an auxin receptor associated with root regeneration ability in tobacco cell suspensions (66) and from the *superroot* (rty) *Arabidopsis* mutants which are auxin overaccumulators, and display large numbers of adventitious and lateral roots, among other auxin related phenotypes (67). The central role of auxin, however, does not mean that these molecules are always a chief factor in rooting; different physiological conditions and genotypes may have different limiting factors.

Auxin metabolism involves at least four components: biosynthesis, conjugation, transport and degradation. Auxin derives from tryptophan, indole glycerol phosphate or indole (68). Conjugates are generally regarded as inactive and can revert to auxin, creating a potential highly effective mode of regulation of auxin activity in the cell. Most conjugates are auxins covalently bound through their carboxyl group to amino acids or peptides (amide link) or to sugars or inositol (ester link). The most common indol acetic acid (IAA) conjugates are with aspartic acid (IAAasp) and glucose (IAAglu). In general, conjugates are regarded as less susceptible to degradation by auxin catabolic enzymes. Conjugates may also function as transport forms of auxins, since some IAA conjugates

have been shown to have much faster transport rates than free IAA (69). According to various reports, auxin catabolism may involve the action of certain peroxidases (E.C. 1.11.1.7) (especially basic isoforms), as well as the co-factors Mn^{+2}, oxygen and monophenolics (70). The simultaneous occurrence of these co-factors and the associated enzymes in the basal portion of cuttings are not difficult to accept, given the effects of cell rupture at the wound site. However, several authors have questioned the role of peroxidases in auxin catabolism because of a lack of significant concentrations of the products of IAA oxidation (namely the loss of carboxyl carbon) in plant tissues, as well as the absence of changes in IAA contents in transgenic tobacco plants expressing high or low peroxidase amounts (68). This last observation could be related to the expression of a peroxidase isoform with low auxin oxidation capacity, since these enzymes are generally encoded by a complex gene family. Evidence in support of alternative non-decarboxylative oxidation pathways, with oxidation in the 2 position or after conjugation with aspartate has been provided, for example, in *Populus* hybrids (71).

Auxin action, like that of all phytohormones, is strongly dependent on adequate interaction with receptor proteins. Auxin receptor density on the membrane and an effective auxin-receptor physical interaction together constitute an additional mode of regulating auxin activity in plant cells. The best characterized auxin receptor is auxin-binding protein 1 (ABP1), a predicted β-barrel dimer with the C-terminus free to interact with other proteins, apparently localized to the endoplasmic reticulum, Golgi and plasma membrane (72). Controlled expression of ABP1 cDNA in leaf strips of tobacco resulted in increased capacity for auxin-mediated cell expansion (even in areas that were previously non-responsive), whereas ABP1 induction in intact plants yielded plants with normal morphology and larger cells; the latter effect was also observed in maize cell lines constitutively expressing the receptor (73). These results show the potential of receptor modification to design plants with better auxin responses, an approach eventually applicable to ARC once auxin receptors involved in root formation are made available and characterized.

Other relevant auxin interacting proteins that have been characterized in detail include the transporters involved in the basipetal flow of auxin from the shoot apex to the root. In roots, both acropetal (towards the root apex) and basipetal auxin movements occur, both movements also mediated by transporters. Acropetal movement of auxin in roots proceeds via the vascular parenchyma cells and basipetal auxin movement in roots occurs through the cortex and/or epidermis. Auxin flow towards the base of cuttings is important for root formation, as discussed earlier. The basipetal movement of auxin in cells of the vascular parenchyma of stems is energy-dependent. It is based on the existence of a pH gradient between the cytosol (pH 7) and the apoplast (pH 5), maintained by proton-pumping ATPases on the plasma membrane, and auxin transporters distributed in asymmetric fashion, particularly at the basal end of the cells. Import of apoplastic IAA into the cell is thought to be favored by the protonated state of the molecule and facilitated by an influx carrier, thought to be encoded by the *AUX1* gene (55,74). At the intracellular pH, IAAH dissociates and efflux of IAA- is strictly dependent on a basal end efflux carrier complex composed of two polypeptides and sensitive to auxin transport inhibitors, such as N-naphthylphtalamic acid (NPA).

The first polypeptide is an integral membrane transporter encoded by one of the members of the *PIN* gene family. The identification of this gene family in *Arabidopsis*

indicates that there are multiple auxin efflux carriers with distinct expression patterns, reflecting the complexity of the control of auxin flow in plant development. The second protein component of the efflux carrier complex is an NPA-binding protein, apparently a peripheral membrane protein that associates with the cytoplasmic face of the plasma membrane, presumably in close proximity to PIN (74). Asymmetric distribution of the efflux carrier complex to the basal portion of plasma membrane and its stabilization seem to depend on actin filaments of the cytoskeleton. The use of kinase and phosphatase inhibitors and the analyses of mutants with defective genes for these enzymes suggest a role for phosphorylation and dephosphorylation in the regulation of auxin transport (74). It has been suggested that the effect of NPA could mimic that of endogenous compounds, such as flavonoids, which inhibit NPA-binding and auxin efflux *in vitro* (75). *Arabidopsis* mutants bearing biosynthetic defects in flavonoid biosynthesis display elevated auxin transport and increased root branching, as expected for plants with higher auxin flow (76). On the other hand, effective adventitious rooting of *Eucalyptus gunnii* was associated with the presence of endogenous flavonoids of the quercetin glycoside type (77). Distribution and accumulation of flavonoids could modulate auxin transport *in vivo*. Structure activity studies on the interaction of specific flavonoid types and NPA-binding protein may help define the effects of these compounds on auxin transport. More recently, evidence for the possible involvement of ABC protein members in auxin transport has been provided by the characterization of the *AtMRP5*, *AtMDR1* and *AtPGP1* genes of *Arabidopsis* (78). The analyses of mutants defective in these genes showed several auxin related phenotypes, indicating that primary active transport (ABC proteins perform ATP-dependent transport in cells) may play a role in auxin or auxin conjugate homeostasis.

Beyond the interaction with its receptors and transport proteins, auxin triggers a signal transduction cascade that leads to modifications in gene expression, eventually culminating with a physiological response. One of the mechanisms of auxin action involves the ubiquitin-proteosome pathway, a complex of proteins that targets cellular proteins to degradation. Genetic studies in *Arabidopsis* indicate that response to auxin is dependent on proteins that function in this pathway. The AUXIN RESISTANT 1 (AXR1) gene, for example, encodes a subunit of an enzyme involved in the activation of an ubiquitin-related protein Rub1 (79). Based on the nuclear localization of some of these gene products it appears that auxin acts by inducing the ubiquitin-proteosome pathway to degrade repressors of the auxin response (*i.e.* DNA binding proteins that block transcription of auxin responsive genes). Primary response genes are rapidly induced by auxin independent of protein synthesis and probably are required for the induction of secondary genes associated with the auxin response. Several primary auxin-response genes can be induced by cycloheximide (a protein synthesis inhibitor), which has been considered as evidence that their transcription might be blocked by short-lived repressors. One of the best known primary auxin-response genes is the *Aux/IAA* gene family, which encodes small short-lived nuclear proteins involved in transcriptional regulation. They are able to bind conserved DNA sequence elements present in promoters of genes responsive to auxin and can form homo and heterodimers, a typical feature of transcription activating proteins (79). The *axr3* semi-dominant mutants of *Arabidopsis* have a constitutive auxin-response phenotype, displaying abundant adventitious rooting, agravitropism, enhanced apical dominance and ectopic expression

of an auxin-regulated gene (80). The *AXR3* gene encodes IAA17, a member of the Aux/IAA family (81). Detailed understanding of the auxin action mechanism, its targets, and the consequent activation of auxin-regulated primary and secondary response genes in *Arabidopsis* (a completely sequenced genome) will pave the way for the search for homologues of these genes in hardwood genomes currently under scrutiny. Modification to components of this protein network may yield interesting phenotypes for tree propagation.

The content of endogenous IAA follows a typical pattern in the several species and culture systems investigated. It is widely accepted that the peak concentration of IAA occurs at the induction phase, whereas the lowest concentration of this phytohormone is detected during root initiation, followed by a new increase as roots grow. The peak concentration of auxin is transient, and sometimes may be observable in a period of as little as 24h (61,82). Several lines of evidence point to an essential role of the auxin peak during root induction: 1) rooting inhibitors also block the auxin increase; 2) the auxin peak is not observable in non-rooting cuttings; 3) the increase in auxin is restricted to the rooting zone; 4) 4-chlorophenoxyisobutyric acid, a competitor for IAA receptors, inhibits rooting without affecting IAA peaking. (61).

The type of auxins and their mode of application may have relevant effects on the rhizogenic response. Most commercial propagation is done by rooting with indole butyric acid (IBA); other auxins often used are IAA and naphthalene acetic acid (NAA). Auxin-type efficacy depends on the affinity for the auxin receptor involved in rooting, on the concentration of free auxin that reaches target competent cells, the amount of endogenous auxin, and on metabolic stability (lower in IAA, intermediate in IBA and higher in NAA) (82). Auxins enter cuttings mainly through the wound surface and appear to be quickly taken up by influx carriers and pH trapping. Auxins may be used transiently at higher concentrations (pulse or sequential medium) or continuously at lower amounts, depending on the rooting protocol. Because of the well-known differential requirements for auxin in the induction and initiation rooting phases, as well as due to economic considerations, it may be advantageous to use IAA, an unstable auxin, to obtain root induction and formation in a single culture medium (with time, auxin catabolism lowers concentrations to an adequate range for root initiation). This has been observed in the rooting of apple microcuttings (82). Other less common endogenous forms of auxin (*e.g.* serotonin) have also been found to be important in the rooting of species such as walnut (61).

In a number of species, peroxidases have been shown to display a pattern of activity during rooting that is typically at a minimum at the inductive phase and maximum at the initiation phase (*e.g.* 83,84,85). Whether these enzymes act in auxin catabolism, or simply constitute markers of the rooting phases, is still not fully established. However, the pattern of peroxidase activity during adventitious rooting seems to be the reverse of that of endogenous free IAA. The auxin resistant, non-rooting mutant of tobacco, *rac* displays higher peroxidase activity (both basic and acidic), higher ethylene production and higher lignification (86). The phenotype seems to be related to growth limitation due to auxin catabolism (by oxidation with increased basic peroxidases) and cell wall rigidification (by enhanced monolignol polymerization via acidic peroxidases), although the relationships between these features and root recalcitrance still need evaluation.

The content of phenolic compounds has also been shown to vary during adventitious rooting, essentially in a way that parallels that of free IAA (the opposite of the variations in peroxidase activity). *In vitro*, phenolics can modulate the activity of peroxidases presumably involved in auxin degradation. Monophenolics act generally as promoters of peroxidase activity, whereas di and polyphenolics act as peroxidase inhibitors (87). More recently, phenolics (e.g. ferulic acid and phloroglucinol) have been regarded as important adjuvants during the first stages of the rooting process (along with other wound related compounds, such as jasmonate and oligosaccharides), potentially enhancing the competence of target tissues for rooting; phenolics apparently act as antioxidants, protecting IAA from oxidation and plant tissue from oxidative stress due to wounding (82).

Ethylene is normally induced by high auxin concentrations through the promotion of transcription of 1-aminocyclopropane-1-carboxylic acid (ACC) synthase gene, and may affect rooting responses (88). Ethylene may also enhance sensitivity to auxins (89). In apple microcuttings, ethylene was promotive to rooting in the first hours after obtaining the cutting and inhibitory during later induction; moreover, well aerated systems such as leaf slices had rooting promoted by providing the ethylene precursor ACC, whereas in agar grown cuttings ACC was inhibitory, presumably due to toxic amounts of ethylene accumulated around the basal stem (82). Equimolar amounts of silverthiosulfate (an inhibitor of ethylene action) and NAA during the induction phase in cuttings significantly improved the rooting response. Ethylene plays a role in the later stages of rooting by inducing acidic peroxidases involved in lignin biosynthesis and cellulases and pectinases that facilitate root emergence through stem tissues (86,90). Ethylene may also promote rooting by stimulating cytokinin catabolism (29). For many woody species, however, ethylene apparently plays a secondary role in ARC.

In many plant developmental processes, auxins are known to interact with cytokinins, often in antagonistic fashion. Applied cytokinins normally act as inhibitors of rhizogenesis, especially when applied early in the process. Cytokinins are also known to stimulate shoot growth. Root tips are considered important sources of cytokinins; these compounds move to the shoot through the transpiration stream. These and other observations lead to the idea that cytokinins may act as endogenous inhibitors of adventitious rooting, counteracting auxin that is basipetally transported from the shoot. Root removal in pea cuttings caused a decrease in the endogenous content of cytokinin; root initiation took place as soon as the cytokinin concentration had fallen below a certain threshold level (91). In this and many other herbaceous species, rooting inhibition by cytokinins constitutes the main limiting factor to ARC rather than promotion of rooting by auxin. Cuttings of *Picea abies* grown under high light intensity (difficult-to-root) showed higher amounts of cytokinins than those grown under low light (easy-to-root) (29). Isolation, partial characterization and bioassays with the purified root inhibitor confirmed it as a putative cytokinin. At later stages of rhizogenesis, a certain amount of exogenous cytokinin may be favorable, since it promotes cell division through the transcriptional induction of cyclins, proteins involved in the control of the cell cycle (92).

Polyamines have been associated with ARC in several species. Detailed studies in the rooting of poplar shoots verified a peak of putrescine (but not of spermidine or spermine, which remained unchanged) close to the IAA peak during root induction.

Supplying putrescine to cuttings prior to or at the beginning of the induction phase stimulated rooting. The rise of putrescine is restricted to basal portions of the cuttings and was absent in non-rooting cuttings. The use of putrescine biosynthesis inhibitors blocked root induction, and metabolic inhibitors that prevented putrescine conversion to spermidine and spermine favored rooting, even in the absence of exogenous auxin. The transient rise and fall of free putrescine and free IAA seem closely related. It has been suggested that both are required for cell divisions at the end of the inductive phase (61).

Besides the mutant studies and the biochemical investigations so far discussed, another tool that can provide information on the genetic and physiological basis of ARC is the infection of plants with the soil bacterium *Agrobacterium rhizogenes*, the cause of the hairy root syndrome in dicots. The phenotype, upon successful infection, is abundant growth of adventitious roots at the site of infection (*e.g.* the base of cuttings). The neoplastic roots are transformed by a fragment of the Ri plasmids. The transferred fragment contains agropine (bacterial food source) and auxin biosynthetic genes; the latter appear to play an accessory role (61). Four open reading frames, designated *rol* A, B, C, D promote the initiation and growth of adventitious roots (93). The *rolB* gene is the most important for the induction of adventitious roots, with other genes playing secondary roles. The mechanism of action of *rolB* involves changes in auxin sensitivity and auxin signal transduction, presumably increasing auxin response at the site of wounding, leading to root formation (94). Application of *A. rhizogenes* infection to root cuttings of difficult-to-root woody species has been generally successful, with development of improved rooting and root systems without morphological abnormalities. Examples include apple (94), plum, *Pyrus pyraster* and almonds (95) and walnuts (96). The use of this bacterium to improve rooting may also reduce costs, as the need for auxins is often eliminated.

In summary, there are many possibilities for improvements in the current clonal propagation methods used for hardwoods. An understanding of adventitious rooting will become increasingly detailed through experimental approaches that range from simply revisiting previous practices, with the various phases of the rooting process and their specific features in mind, to the definition of specific gene activation programs that lead to the expression of a new root system. This last acquisition is anchored on the functional and full genome sequencing projects in hardwood species, the availability of methods to monitor multiple gene expression profiles (by differential display and DNA arrays) and the discovery of gene functions related to rooting and auxin physiology in the model plant *Arabidopsis thaliana*, gathered from its vast mutant collection and from its fully sequenced genome. Important genes in *Arabidopsis* rooting and auxin activity will continue to provide sequences for the search for homologues in gene databases fed by tree sequencing projects. Similar impacts will likely be seen in somatic embryogenesis methods and on the process of *Agrobacterium rhizogenes*-induced rooting.

The usefulness for tree physiologists of genes controlling development in *Arabidopsis thaliana* (Brassicaceae) must not be underestimated, and is well established in the flowering literature. Genes controlling floral meristem identity and floral homeotic genes first characterized in *Arabidopsis* have found numerous homologues in tree species. Constitutive expression of the *Arabidopsis* floral meristem identity gene *LEAFY* in poplar produced flowering in only six months, with interesting implications for

breeding programs (97). Successful acceleration of flowering and shortening of the juvenile phase was achieved by constitutive expression of the *Arabidopsis LEAFY* or *APETALA1* (also involved in meristem identity) genes in *Citrus* sp., leading to flowering in consecutive years (starting at the first year of age) and under environmental control (98). The use of inducible and tissue specific promoters to drive expression of such genes in trees are technically available refinements that are still little explored. The possibility of the existence of master genes regulating adventitious rooting is an exciting thought. It is just a matter of time before progress in the knowledge of adventitious rooting (at the genetic, molecular and physiological levels) can produce impacts on the mass clonal propagation of hardwoods.

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