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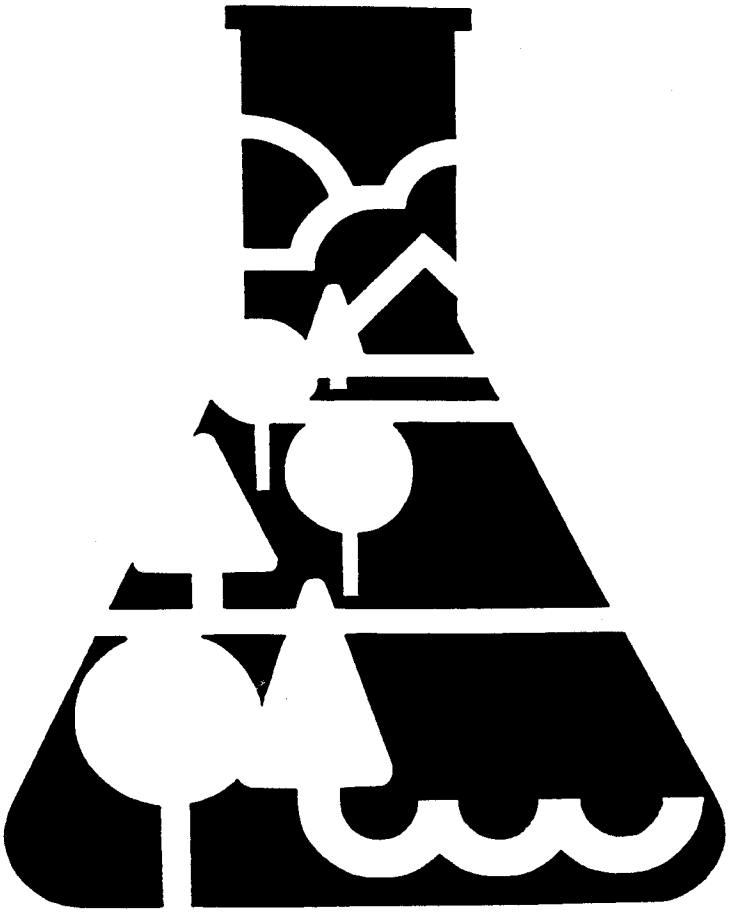
July 8-10, 1992

"Applications of Vegetative Propagation in Forestry"



Proceedings of the Southern Regional Information Exchange Group Biennial Symposium on Forest Genetics

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and

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PREFACE

This volume is the proceedings of the 1992 Southern Regional Information Exchange Group (SRIEG) Biennial Symposium on Forest Genetics. The meeting was held in Huntsville, Alabama, USA on July 8-10, 1992 and was entitled, "Applications of Vegetative Propagation in Forestry." The nine papers were divided into three sets under the headings: "Tissue Culture," "Rooted Cuttings," and "Propagule Growth, Development and Applications." The authors of each paper were given the task of synthesizing the current state of knowledge for their appointed topic. The meeting was co-hosted by SRIEG, the USDA Forest Service Southern Forest Experiment Station, and Alabama A&M University. This proceedings may be referenced as a 1993 publication.

The Southern Regional Information Exchange Group is sponsored by the University Experiment Stations in the Southern Region.

The editors, G. Sam Foster and Alex M. Diner, express their gratitude to Lyn Thornhill, who compiled and formatted the proceedings into their final publication form. We also thank the authors for their major effort in writing the manuscripts and giving the presentations at the Symposium.

Special thanks go to the moderators for the symposium: Henry V. Amerson, Tissue Culture; Hans van Buijtenen, Rooted Cuttings; and Barbara McCutchan, Propagule Growth, Development and Application. Their participation greatly enhanced the symposium.

Members of the Steering Committee are acknowledged for their tireless and superb help in the local arrangements for the Symposium:

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Tissue Culture

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TISSUE CULTURE MICROPROPAGATION OF CONIFERS[1]

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Abstract. The future of micropropagation technology is bright with respect to its immediate utility as an important tool in tree breeding programs and to its use as a mass production methodology of germplasm produced by traditional and biotechnological means. In general there are three broad regenerative pathways that allow the in vitro vegetative propagation of conifers: (1) regeneration through somatic embryogenesis, (2) regeneration from adventitious meristems, and (3) regeneration via proliferation or enhancement of bud break of existing meristems. This paper outlines the current understanding of the major in vitro propagation strategies based upon these three regenerative pathways and discusses some of the key challenges that remain to be addressed.

INTRODUCTION

Significant progress has been made in exploring opportunities for the use of in vitro-propagated conifers in forestry since the first report of plantlet regeneration in *Pinus palustris* Mill via organogenesis from mature zygotic embryos (Sommer et al. 1975). Since this first report there has been a proliferation of micropropagation technologies and applications of those technologies that offer considerable promise for use in the forests of the world. These technologies encompass an array of techniques designed for the "true-to-type" propagation of selected genotypes (Debergh and Read 1991).

Assessment of the progress and significance of the application of micropropagation technologies to the vegetative regeneration of forest trees has been the subject of numerous publications (Boulay 1987a,b, Gupta 1988, Thorpe et al. 1991, von Arnold 1991, Zobel 1992). Zobel (1992) concluded that "Currently, the most promising use for tissue culture and biotechnology is to supply desired genotypes developed by the tree breeder, in the proper physiological stage, so that they can be reproduced in mass using conventional rooted cutting methodology." Similarly, von Arnold (1991) suggested that the contribution of in vitro vegetative propagation to the improvement of forest trees included the "exploitation" of existing "plus" genotypes and the production of commercially valuable new genotypes. Boulay (1987a) also emphasized the importance of clonal forestry, but discussed problems implementing these new technologies on an operational scale. He suggested that the slow incorporation of in vitro methodologies, for vegetative propagation on a massive scale, was

[1]Paper presented at the 1992 Southern Regional Information Exchange Group Biennial Symposium, Huntsville, AL, July 8-10, 1992.

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the result of resistance from forest nursery operations because of past orientation to seedling production and lack of experience in the production of rooted cuttings. The low cost of forest tree seedling production, coupled with the lack of elite clones and numerous technical difficulties was also said to contribute to the slow acceptance of these technologies. Despite known problems and those not yet encountered, the future of micropropagation technology appears to be bright with respect to its immediate utility as an important tool in tree improvement programs and to the promise for its use as a mass production methodology of elite germplasm.

The selection of a specific approach for in vitro vegetative propagation of conifers is often based upon objectives such as the development of totipotent cellular regenerative systems designed as a vehicle for gene insertion for the eventual production of transgenic plants, the propagation of valuable, improved germplasm to allow field testing across broad environmental gradients, or the mass production of proven (mature) genotypes for large operational plantings (Zobel 1992). The attainment of any of these objectives requires overcoming numerous biological barriers established through millennia of evolutionary trial and error. The basic problem faced by researchers is to overcome and successfully manipulate the innately programmed developmental sequencing responsible for the production of the normal phenotype.

It is generally agreed that there are three different regenerative pathways (von Arnold 1991, Gupta 1988) used for the in vitro vegetative propagation of conifers. The pathways are (1) regeneration through somatic embryogenesis, both direct and indirect; (2) regeneration from adventitious meristems; and (3) regeneration via the proliferation or enhancement of axillary bud development of existing meristems. All of these propagation pathways are currently being investigated worldwide by academic and industrial research groups. The literature contains numerous publications surveying various aspects of these micropropagation technologies. Recent papers include reviews by Boulay (1987b), Fowke and Hakman (1988), Mehra-Palta and Thompson (1988), Thorpe (1990) and Thorpe et al. (1991). This paper presents an overview of the current understanding of the in vitro propagation strategies used in the three regenerative pathways listed above and discusses some of the challenges that need to be addressed in order to allow widespread use of micropropagation technologies in forestry.

REGENERATION THROUGH SOMATIC EMBRYOGENESIS

Somatic embryogenesis has been defined as a "non-sexual developmental process which produces a bipolar embryo from somatic tissue" (Tulecke 1987). There is some confusion in the scientific literature as to which developmental processes are encompassed in each stage, e.g., maturation. To facilitate accurate interchange of information among scientists, it would be beneficial to develop a common definition of terms for the substance and process of the morphogenesis of fully "mature" somatic embryos and for the developmental processes of germination and early growth of the newly regenerated plantlet. The generation of too many "new" terms is not a concern, as long as they are precisely defined and uniformly applied. The following describes a sampling of the current nomenclature and some areas of possible confusion.

Systems for tracking the ontogeny of somatic embryo maturation and germination have been described (Dunstan et al. 1988, Hakman and von Arnold 1988, Webb et al. 1989). The morphogenic process is usually divided into four stages, each stage being defined by a more or less clearly observable set of morphological characteristics. For example, Tautorus et al. (1991) provides a clear definition and illustration of each of the four stages (stage 1 through 4b) for the maturation of *Picea mariana* somatic embryos into plants. Nomenclature used by Webb et al. (1989) was based upon the classification system for the development of zygotic embryos by Buchholz and Stiemert (1945). This nomenclature describes, in four discrete stages, the maturation of the somatic embryo but, contrary to Tautorus et al. (1991), excludes "plantlet" attainment.

The final two steps, maturation and germination, are reasonably well demarcated through an increase in morphological complexity to produce an anatomically complete and physiologically mature embryo that resembles its zygotic counterpart. The maturation process should ideally end with the production of an embryo that, given the appropriate environmental cues, is capable of immediate germination, a developmental state not unlike that exhibited by the embryo in a fully mature "quiescent" seed (Roberts et al. 1990a). These last

two steps in the production of plantlets have been variously described by different authors in attempting to detail the various developmental hurdles presented by the sometimes recalcitrant cultured embryos. Becwar et al. (1989) suggested that the process of regeneration via somatic embryogenesis be divided into three developmental stages: "(1) 'maturation' representing the growth of immature somatic embryos to the cotyledonary stage, (2) 'germination' as evidenced by growth of the radical, and (3) 'conversion' indicating the survival and continued growth of in vitro-derived plantlets in soil." Gupta et al. (1991) define the term "conversion" as the "...process of turning somatic embryos into plants growing autotrophically in soil." The term is further described to include the germination of a "well formed embryo" on a germination medium to facilitate the development of true leaves followed by successful outplanting in soil. It is also stated that to attain a reasonably high level of conversion one must start with a fully developed and mature embryo.

Although there is not total agreement about all of the details of the ontogeny of somatic embryos (Becwar et al. 1991, Gupta and Durzan 1987a), most authors agree that somatic embryos are produced by a recapitulation of development closely paralleling the genesis of a normal zygotic embryo. Sharp et al. (1980) describe two distinct routes to somatic embryo formation, direct and indirect. The direct developmental route is defined as the formation of a new somatic embryo either from explant tissue without an intervening callus or free-cell stage, or from a previously formed somatic embryo. The somatic embryo develops directly from the explant tissue as a product of a single cell or of a small group of totipotent vegetative cells. The production of embryos by indirect somatic embryogenesis proceeds via callus or cell suspension, or via a single cell or group of cells derived from existing somatic embryos (Tautorus et al. 1991). However, according to Williams and Maheswaran (1986), "Once induction of embryogenic determined cells has been achieved, there appears to be no fundamental differences between indirect and direct somatic embryogenesis."

Production of embryos via direct or indirect somatic embryogenesis can affect the genetic fidelity of the resulting clonal regenerants (Berlyn et al. 1986). The use of a direct somatic embryogenic pathway may present several advantages, as the presence of a callus stage in a propagation strategy has a greater probability of introducing genetic, developmental, and physiological instability into the cloned germplasm (Chaleff and Keil 1981, Larkin et al. 1989). Berlyn et al. (1980) suggested that "Genetic changes caused by culture conditions and undetected in tissue culture-derived plants could lead to large investments in forests of defective trees." This possibility emphasizes the need for screening for genetic defects before the germplasm is released for plantation deployment (Becwar et al. 1991, Berlyn et al. 1986). Somaclonal variations were found in *Picea abies* globular somatic proembryos and mature somatic embryos obtained from immature embryos and cotyledons of young seedlings (Lelu 1988). A positive note can be sounded for the genetic stability of embryogenic cultures of *Picea glauca* x *engelmannii* complex (Eastman et al. 1991). After extensive isozyme analysis and tracking of culture morphology, the authors concluded that "...embryogenic cultures initiated from interior spruce embryos show a high degree of genetic stability in that the morphological behavior and isozyme phenotype were always consistent with that of the explant genotype."

The successful establishment of an efficient somatic embryogenic propagation system for forest trees is predicted to have many advantages (Cheliak 1991, Durzan and Gupta 1988, Gupta et al. 1991, Thorpe et al. 1991) for all aspects of forestry. According to Thorpe et al. (1991), the difficulty and time required to induce rooting of shoots produced through the various organogenic-based methodologies is eliminated, because a mature somatic embryo comes with complete bipolar development of apical and root meristems. Somatic embryogenesis offers an efficient method for the production of large numbers of "emblings" (Libby 1986) through the development of automated, large-batch, cell suspension cultures (Durzan and Durzan 1991). In addition, there is the opportunity for the application of genetic engineering technologies to embryogenic protoplasts derived from embryogenic cells and tissues (Gupta et al. 1988, Wilson et al. 1989) in order to produce transgenic plants.

Induction

The "process" of in vitro plantlet regeneration via somatic embryogenesis has been described as the (1) induction and/or initiation of embryogenic callus on the explant, (2) proliferation of the resulting embryogenic tissue, (3) maturation of the somatic embryos, and (4) germination of the fully mature somatic embryos (von Arnold 1991). The first step in the regeneration process, the induction of the embryogenic state in the explant, requires the

redirecting or resetting of the developmental program controlling the morphogenic fate of the targeted cells. It is unknown to what extent this involves a relatively minor developmental redirection, which may be the case for the embryogenic callus protruding from the micropylar end of a female gametophyte (Becwar et al. 1990, Finer et al. 1989), or a major resetting and redirecting of the morphological endpoint of the explant, as might be the case for the induction of embryogenic callus from developing young seedling tissue (Attree et al. 1990a, Mo and von Arnold 1991). Even in the same species, newly induced embryogenic tissue/callus can vary slightly in cellular morphology with respect to the presence or absence of well-formed somatic embryos (Becwar et al. 1990, Gupta and Durzan 1987a,b). In general, however, the standard description for conifer embryogenic tissue is a glossy, translucent, white, mucilaginous cellular mass. This tissue contains a variable mix of elongated cells, aggregates or clumps of densely cytoplasmic cells, embryo initials, and sometimes later stage embryos (Finer et al. 1989, Gupta and Durzan 1986, Gupta and Durzan 1987a, Hakman et al. 1985, Laine and David 1990, Lu and Thorpe 1987).

The initiation of the embryogenic state in explant tissue is reasonably complex, as it involves an interplay of variables that include, but are not restricted to, explant age and physiological condition, incubation medium, and environment. The choice of a specific combination of these variables is increasingly based upon a review of the literature of related systems and a growing understanding of the physiology and biochemistry of the embryogenic process. Comparative studies of media composition, genotype, and culture conditions have all contributed to an understanding of the importance that each plays in the initiation process (Attree et al. 1990a, Becwar et al. 1990, Hakman and von Arnold 1985, Jain et al. 1989, Kvaalen and von Arnold 1991, Roberts et al. 1989, Tautorus et al. 1990, Tremblay 1990, Webb et al. 1989). Continued research is being focused on discovering and then optimizing the factors that promote the induction of somatic embryogenesis. These include the selection of the optimum developmental stage of the explant donor, the selection of the culture medium plus hormone additives, and the provision of appropriate environmental conditions for culture development (Tulecke 1987, Webb et al. 1989). Tautorus et al. (1991) have recently published an excellent review of the "most recent directions in research of conifer embryogenesis." These authors present a tabulation of conifer species that have produced somatic embryos. Six genera of conifers are represented *Abies*, *Larix*, *Picea*, *Pinus*, *Pseudotsuga*, and *Sequoia*. Twenty species and species complexes are included, with the genera *Picea* and *Pinus* each having six species represented.

The explant tissues used to initiate somatic embryogenesis encompass a range of developmental states, including reproductive generative structures (megagametophytes containing the developing zygotic embryo) (Becwar et al. 1990, Finer et al. 1989, Nagmani and Bonga 1985), immature and mature zygotic embryos (Jain et al. 1989, Wann et al. 1989, Webb et al. 1989), hypocotyl and cotyledonary tissue from seedlings (Becwar et al. 1988, Krogstrup 1986, Lelu et al. 1987), and recycled cotyledonary somatic embryos (Eastman et al. 1991, Mo et al. 1989). The majority of investigators use immature to mature zygotic embryos as the explant to study induction of embryogenic callus in conifers (Durzan and Gupta 1988, Tautorus et al. 1991). Webb et al. (1989) studied the relationship between the efficiency of embryogenic callus induction and the production of shoot-forming callus (i.e., caulogenesis) in *Picea glauca* and *P. engelmanii* as related to the degree of zygotic embryo maturation. They found that the stage of active cotyledonary development was most competent for embryogenic callus induction, in agreement with several previous reports for spruce zygotic embryos (Becwar et al. 1988, Hakman and Fowke 1987, Lu and Thorpe 1987). Interestingly, they also reported that competence for caulogenesis seemed to be inversely related to the degree of maturation of the spruce zygotic embryos. Becwar et al. (1988) compared several species of *Picea* and *Pinus* with respect to the optimal developmental stage for initiation of embryogenic tissue. They concluded that pre-cotyledonary embryos were optimal for *Pinus* species and that initiation in *Picea* was most efficacious when post-cotyledonary embryos were targeted for explant use. In addition, the origin of the embryogenic tissue differed between the two genera. In the three *Pinus* species, *P. serotina*, *P. strobus*, and *P. taeda*, embryogenic tissue seemed to originate from the suspensor region of the precotyledonary embryo. In contrast, the hypocotyl and cotyledons of more mature post-cotyledonary zygotic embryos of *Picea* produced the embryogenic tissue.

As knowledge of the basic culture requirements for induction of somatic embryogenesis from immature tissues has increased, parallel successes

have been achieved in chronologically older and, consequently, morphologically more advanced explant tissues. Mature zygotic embryos removed from seed that had been in long term cold storage have produced embryogenic callus (Tautorus et al. 1990, Tremblay 1990). Tremblay (1990) succeeded in regenerating plantlets from embryogenic callus of *Picea glauca* produced from mature zygotic embryos that had been stored for 3 to 11 years.

The induction of embryogenic callus was reported for young seedling material of *Picea* (Attree et al. 1990a, Krogstrup 1986, Mo and von Arnold 1991). Embryogenic callus was induced on 12-day-old seedling tissues of *Picea mariana* grown from seed that had been stored for 10 years (Attree et al. 1990a). These calli were established in liquid suspension culture and subsequently transferred to a maturation medium on rigid filter paper supports. As embryos matured, they were separated from the main callus mass in order to facilitate continued development. One hundred and eleven plants were recovered, transferred to pots, and moved to a growth chamber.

Proliferation

Embryogenic callus, or perhaps more correctly embryogenic tissue (*sensu* Tautorus et al. 1991), is used to accomplish the second stage of in vitro plantlet regeneration, "proliferation." At this stage, alterations can be made in protocols to foster different proliferation methods. Several options are available for multiplication of embryogenic tissue. Multiplication of the existing tissue can be achieved in its initial morphological state. Depending upon the species and genotype, embryogenic tissues can continue to be grown on the induction medium (Becwar et al. 1990, Finer et al. 1989, Jain et al. 1989, Mo et al. 1989, von Arnold and Woodward 1988, Webb et al. 1989) or they may require a medium reformulation (Becwar et al. 1990, Laine and David 1990). Proliferation of the embryogenic tissue can also take place in liquid suspension cultures. The advantages of this approach to somatic embryo production have been amply discussed (Becwar et al. 1988, Dunstan 1988, Durzan and Gupta 1988, Gupta et al. 1991). Liquid suspension culture of embryogenic tissue provides an opportunity for enhanced growth rates over growth on solid medium, providing an avenue for the economical production of trees for reforestation (Lulsdorf et al. 1992). Culture variables that influence growth rate and overall culture productivity in liquid systems are now being elucidated. Krogstrup (1990) described the relationship between suspension culture density and proliferation rate of stage 1 somatic embryos of *Picea sitchensis* initiated from mature zygotic embryos. Dunstan (1988) maintained embryo cultures of *Picea glauca* for extended periods of time through subculture in liquid medium. These cultures yielded 150 to 300 stage 1 embryos per milliliter of culture medium, with a 3-to 5-fold multiplication every 7 days. Although the embryo productivity levels demonstrated were still not at levels required to fill even a small part of the yearly seedling requirement for reforestation of any commercial species, this methodology demonstrates the potential for the development of large-scale bioreactor production of somatic embryos. Tautorus et al. (1991) lists eight species of conifers that have been established from embryogenic tissue in shake-flask suspension cultures (*Abies nordmanniana*, *Picea abies*, *Picea glauca*, *Picea mariana*, *Pinus caribaea*, *Pinus strobus*, *Pinus taeda*, and *Pseudotsuga menziesii*).

Suspension cultures of embryogenic cells have been used as a source of protoplasts that have been regenerated into somatic embryos. Regeneration of somatic embryos from protoplasts was first reported for *Pinus taeda* (Gupta and Durzan 1987b) and *Picea glauca* (Attree et al. 1987) and later for *Pseudotsuga menziesii* (Gupta et al. 1988), *Picea mariana* (Tautorus et al. 1990), *Pinus caribaea* (Laine and David 1990), and haploid *Larix decidua* (von Aderkas 1992). Plantlet regeneration from protoplast cultures has been achieved in *Picea glauca* (Attree et al. 1989) and *Larix* x *eurolepis* (Klimaszewska 1989). The regeneration of somatic embryos and then plantlets from protoplasts may provide the necessary vehicle for the application of genetic engineering to conifers (Tautorus et al. 1991).

Maturation

The process of somatic embryo maturation, broadly defined as the production of embryos physiologically and morphologically capable of germination and early development, is currently poorly understood. The efficiency of the production of embryos to this "mature" developmental state has been relatively low (Dunstan 1988, Gupta and Durzan 1987a, Hackman and von Arnold 1988, von Arnold 1987) and varies with plant genotype (Jalonen and von Arnold 1991, Webster et al. 1990). Considerable improvement in the efficiency of bringing somatic embryos to the mature state has

resulted through the use of abscisic acid (ABA) (Becwar et al. 1989, Dunstan et al. 1988, Dunstan et al. 1991, Durzan and Gupta 1987, Gupta and Durzan 1987b, Roberts et al. 1990a, von Arnold and Hakman 1988), partial desiccation at high relative humidity (Roberts 1991, Roberts et al. 1990b), and manipulation of the gaseous environment of the culture vessel (Kvaalen and von Arnold 1991).

Somatic embryos are usually proliferated on medium containing various levels of growth regulators, after which they are transferred to medium with a reduced level of hormone and containing ABA (Attree et al. 1990b, Atree et al. 1992, Dunstan et al. 1988, Roberts et al. 1990a,b). In general, the combination of the reduced growth regulator levels, a brief exposure to ABA, and partial drying at high relative humidity stimulates development of fully mature embryos. The importance of using the appropriate level of ABA in the medium during the maturation process was emphasized by Roberts et al. (1990a). The maturation pattern of ontogenetic development followed by somatic embryos of *Picea glauca* x *engelmannii* hybrids was dependent upon the media concentration of ABA. Without ABA, little or no development occurred, and a range of embryo morphologies were produced as the ABA concentration of the medium was increased. Low levels of ABA (1 to 10 μM) produced "shooty embryos;" intermediate levels (10 to 20 μM) produced bipolar embryos that often precociously germinated, whereas 30 to 40 μM of ABA produced opaque cotyledonary embryos that entered a quiescent stage of arrested development. When indolebutyric acid (IBA) was added to the ABA-containing medium, the number of mature embryos was significantly increased. Incorporation of ABA and IBA into the medium resulted in a developmental synchrony in the somatic embryos similar to that found in the maturation and germination of naturally produced zygotic embryos (Roberts 1991). Somatic embryos generated through this protocol began to accumulate proteins (Roberts 1991) and lipids (von Arnold and Hakman 1988) in a manner similar to that of maturing conifer zygotic embryos (Feirer et al. 1989).

Germination

The final step in plantlet production, "germination," followed by successful early growth (i.e., conversion [Becwar et al. 1989]), has operationally been somewhat better defined and separated from maturation through the application of ABA and the partial drying step mentioned above. Somatic embryo germination has been variously described as "root and hypocotyl elongation" occurring in a synchronized manner (Roberts et al 1990b), as "...the transition from mature somatic embryos to green plantlets and involves clearly visible root as well as cotyledon and hypocotyl elongation" (Attree et al. 1990b), or simply as "radical growth" (Becwar et al. 1989). The methods used to encourage germination of somatic embryos usually involved transfer of mature embryos to a germination medium lacking growth regulators (Attree et al. 1990b, Mo and von Arnold 1991, Roberts et al. 1990b) with or without a pregermination desiccation treatment (Roberts et al. 1990b). Becwar et al. (1989) found that the placement of mature somatic embryos on the support medium was critical to the efficiency of germination. The highest percent germination, 56% for *Picea abies*, resulted when the cotyledons and apical meristem were inserted in agar with the hypocotyl and radical end projecting downward into the air inside an inverted vessel. The mature embryos were sometimes singulated (i.e., removed from the whole calli) and cultured in individual vessels to enhance germination efficiency (Attree et al. 1990b, Becwar et al. 1989, Roberts et al. 1990b).

Few studies describe the moderate- to large-scale soil establishment and subsequent long-term field performance of emblings. Becwar et al. (1989) reported 2-year survival of six phenotypically normal *Picea abies* emblings. Webster et al. (1990) presented a detailed study of the propagation and regeneration of 71 genotypes of embryogenic cultures of *Picea glauca* x *englemanii* obtained from immature embryos. They reported an 80% or greater survival rate of emblings for most genotypes after one season's growth under nursery conditions. Their results suggested that "...somatic embryogenesis can be used for production of planting stock for a range of interior spruce genotypes."

REGENERATION FROM ADVENTITIOUS MERISTEMS

Clonal propagation via adventitious meristems involves the induction of unipolar shoots (Thorpe et al. 1991) on explants followed by shoot excision and induction of root meristems. Because of the similarity in methodologies used to initiate adventive root production on microshoots produced via both adventitious meristems and axillary buds and their subsequent acclimation to the ambient

environment, a combined discussion addressing rooting and plantlet acclimation is presented in this paper following the section on "Regeneration From Axillary Buds." In conifers, the process of adventive meristem production can follow two major developmental sequences: direct or indirect organogenesis (Hicks 1980).

Indirect organogenesis occurs when there is an initial production of callus tissue from the primary explant, followed by the appearance of meristemoids (Torrey 1966). It is believed that these meristemoids are, at first, not determined (i.e., capable of producing either shoot or root primordia). Progress toward plantlet regeneration in conifers via this methodology has been limited. However, initiation of shoot meristems via indirect organogenesis from callus has been reported (Ball 1950, Gladfelter and Phillips 1987, Kaul and Kochhar 1985, Konar and Singh 1980, Simola and Honkanen 1983, Winton and Verhagen 1977). A low frequency of plantlet regeneration has been achieved in *Larix* x *eurolepis* (Laliberte and Lalonde 1988) and *P. eldarica* (Gladfelter and Phillips 1987) from buds initiated from long-term callus cultures.

The process of direct organogenesis is defined as organ formation directly from the primary explant in the absence of an intervening callus stage (Hicks 1980). The predominant morphogenic route reported for adventitious shoot formation in conifers has been through direct organogenesis without the involvement of an intermediate callus stage (Thorpe and Biondi 1984). In a comparative study of six species from two genera of conifers, Thorpe and Patel (1986) described organogenesis as the formation of meristematic centers or meristemoids which developed into bud primordia and "...finally adventitious shoots with apical domes and needle primordia."

As noted in the introduction, the first complete plantlet regeneration of a conifer (*Pinus palustris*) was accomplished by Sommer and Brown (1974) and Sommer et al. (1975). The buds were produced on the cotyledons of mature zygotic embryos, presumably via a direct organogenic route. The step-wise procedure used in their studies is typical of methods used to induce direct organogenesis in conifers. The procedure involved transfer to several different media in order to induce, first, apical bud initiation, followed by induction of root primordia, and finally, continued plantlet development. Mature embryos were excised from seed and placed on a basal medium containing both an auxin and cytokinin. During the next four to six weeks, areas on the zygotic embryos' cotyledons differentiated shoot buds. These areas were subsequently excised and transferred to a second medium for further development into plantlets. The authors concluded that, in order to facilitate plantlet regeneration, differentiated shoot apices should be transferred to a medium capable of inducing root formation. A final transfer to yet another medium was required to facilitate root elongation and further plantlet development.

Shoot apices, needles, hypocotyls, epicotyls, cotyledons, zygotic embryos, dormant buds, needle fascicles, and lateral buds have been used as explants for the induction of adventitious bud meristems (John 1983, Thorpe and Biondi 1984). Ideally, explant material should come from genetically superior trees, proven by or selected from progeny tests. However, there is a general recalcitrance of explants derived from older, more mature trees to form adventitious buds and roots (Bonga 1987, Durzan 1984, Hackett 1987). The majority of successful reports of plantlet regeneration in conifers have resulted through the use of juvenile explant material.

The procedure typically followed to accomplish plantlet regeneration by direct organogenesis can be divided into four steps: (1) initiation of shoot meristems, (2) development and elongation of shoot buds, (3) root meristem initiation and development, and (4) plantlet acclimation to the ambient environment (Thorpe and Biondi 1984, Thorpe et al. 1986, Thorpe et al. 1991). The details of each of the procedural steps required to accomplish plantlet regeneration vary somewhat depending upon the species of conifer and the type and developmental stage of the explant. What follows is a brief summary of the process from shoot initiation to the production of a shoot ready for rooting. A discussion of the rooting and acclimation procedures required to produce plantlets is presented after the section on "Regeneration From Axillary Buds."

Shoot initiation

Adventive shoot meristems can be initiated by the application of a cytokinin (benzylaminopurine [BA], isopentenyl adenine, kinetin, etc.) (Webb et al. 1988) alone, or in combination with an auxin and/or other plant growth regulators (Chang et al. 1991, Sen et al. 1989) over a range of concentrations, exposure times, and delivery methods (Bonga and Durzan 1987, Thorpe and Biondi 1984). Extensive

experience with a range of coniferous species has indicated that maximum bud yields are obtained through a specific combination of explant and cytokinin-containing medium (Bornman 1983, Thorpe and Patel 1986, von Arnold and Eriksson 1979, von Arnold and Hawes 1989). Typically, a single application of BA at about 25 μM (Thorpe et al. 1991) will produce the desired effect. Abscisic acid (ABA) has been found to enhance shoot formation on loblolly pine cotyledonary explants via the organogenic process (Sen et al. 1989). When ABA was added to an induction medium containing both BA and naphthaleneacetic acid (NAA), both the shoot number and average fresh weight of loblolly pine microshoots increased significantly.

Explant cells responding to the hormone treatment undergo transformation into meristematic centers or meristemoids that ultimately produce buds (see discussion above). Although there are some exceptions, most plantlet regeneration systems rely on a timed exposure to the hormone-containing medium followed by explant transfer to a hormone-free medium designed to promote further development of the newly induced buds (Bronson and Dixon 1991, Jang and Tainter 1991).

A notable exception to this approach is a method described by Aitken-Christie et al. (1988) that relies on BA for initial induction of meristematic tissue followed by continued long-term exposure to the growth regulator. Mature zygotic embryos were cultured on medium containing BA, with subculturing every 21 days for 12 weeks. During this time, the embryos produced "pieces" of meristematic tissue that were easily dislodged from the explant. Subsequently, all tissues were subcultured every 4 weeks to fresh medium containing BA. The "pieces" of tissue were meristematic nodules capable of being maintained and multiplied for extended periods of time. A single embryo of *Pinus radiata* was reported to have produced 5480 pieces of nodular tissue after 13.5 months in culture. Each piece of nodular tissue had the potential of producing an average of 68 elongated shoots. The authors estimated that "...260,000 trees could be produced from a single, good-reacting seed in 2.5 years." It is clear from the description of this regeneration system that there is a need for its application to the propagation of other coniferous species where limitations exist in the numbers of clonal propagules producible per genotype.

Shoot Elongation and Development

The development and elongation of buds, resulting from the production of cell division centers or meristemoids, is usually accomplished by transferring the hormonally treated explant to a medium without hormones. Activated charcoal may or may not be added to the hormone-free medium as required to enhance elongation (Thorpe and Biondi 1984, Thorpe et al. 1991). The time required to obtain shoots of sufficient size (i.e., usually >5 mm in length) for excision and rooting was highly variable. The required development and elongation may be achieved from a single transfer to basal medium for 6 to 10 weeks as with buds induced on cotyledonary explants of *Pinus oocarpa* (Franco and Schwarz 1985) and loblolly pine (*P. taeda*) (Amerson et al. 1988, Mott and Amerson 1981). Sequential transfers to fresh basal medium, however, may be required to produce rootable shoots as in *Picea glauca* and *P. mariana* (Rumary and Thorpe 1984). In *Pinus oocarpa*, the yield of rootable shoots can be increased by subculturing the remaining explant tissue, after removal of the larger shoots, in the same basal medium to allow elongation of the remaining smaller shoots and development of new adventitious buds (Franco and Schwarz 1985). In general, larger shoots tend to root at a higher percentage than smaller shoots produced on the same explant.

Shoot yield can be significantly increased by "hedging" or removal of the top portion of the shoot and reculturing both the severed top and base (Aitken-Christie and Thorpe 1984). In response to the removal of the apical meristem, axillary bud break is stimulated in the basal portion of the shoot (see discussion in the following section). This technique was used by Aitken-Christie and Thorpe (1984) in combination with an adventive organogenic procedure in *Pinus radiata* to produce large numbers of rootable axillary shoots in 3-month cycles and resulted in at least a four-fold multiplication for every cycle.

REGENERATION FROM AXILLARY BUDS

Micropropagation utilizing axillary meristems differs from adventive shoot production only in the ontogenesis of shoot production. After axillary bud stimulation and elongation, rooting and acclimation procedures are essentially the same as those used with shoots of adventive organogenic origin.

Stimulation of axillary and fascicular meristem development is accomplished by hormonal treatment of the explant tissue. Explants have been obtained from juvenile to mature conifers, and regeneration has been achieved in several *Pinus* species (Table 1).

Table 1. Selected list of publications highlighting in vitro microshoot production from axillary and fascicular bud meristems.

Pinus Species	Explant Material	Authors/Year
Pinus brutia Ten.	2-month-old seedling tips	Abdullah et al. (1984)
	6-month-old seedlings	Abdullah et al. (1986)
Pinus eldarica Medw.	seedling shoot tips	Gladfelter and Phillips (1987)
	seedlings	Wagley et al. (1987)
Pinus elliottii Engelm.	30- to 40-day old seedlings	Burns et al. (1991)
Pinus nigra Arnold	3-week-old seedling shoot tips, 1- and 2-year-old resting vegetative buds	Jelaska et al. (1982)
	20-day-old seedlings	Kolevska-Pletikapic et al. (1983)
Pinus oocarpa[1] Schiede	distal portion (10mm) of hypocotyl with cotyledons attached (newly germinated seedling)	Baxter et al. (1989)
Pinus pinaster Aiton	seedling stem apices	David and David (1977) David et al. (1978)
Pinus radiata D. Don	seedling shoot tips	Horgan and Aitken (1981)
	adventitious clonal shoots	Aitken-Christie and Thorpe (1984)
	in vitro hedges	Aitken-Christie and Jones (1987)
Pinus strobus L.	1- to 2-week old seedlings	Kaul (1987)
Pinus sylvestris L.	2-week-old seedling plumules	Bornman and Jansson (1980)
	seedling apical segments	Skripachenko (1982)
	seedling apices	Zel et al. (1988)
	2- to 17-week-old seedling apices	Toribio and Pardos (1989)
Pinus taeda L.	2- to 3-year-old plants	Stomp (1985)
	11-year-old hedges	Amerson et al. (1988)

[1]also *Pinus caribaea* var. *hondurensis* Morlet and *Pinus tecunumanii* Equiluz and Perry (*Pinus patula* Schiede and Deppe ssp. *tecunumanii* [Equiluz and Perry])

Axillary shoots produced in *Pinus* species generally arise from preexisting quiescent meristems. These meristems are located adaxial to juvenile needles, in the axils of cotyledons, in the normally dormant short-shoot surrounded by needle primordia that make up a fascicular budlet, and primarily, in older plant material, adaxial to scale leaves (cataphylls) (David 1982, Lanner 1978, Stomp 1985, Toribio and Pardos 1989). The procedure required to regenerate plantlets utilizing these axillary and fascicular meristems parallels that used in adventive shoot production. The basic steps are (1) stimulation of meristem development, (2) elongation of the developing shoots, (3) induction of adventitious roots, and (4) acclimation of the regenerated plantlet to the ambient environment. Because of the size and vigor of the microshoots produced by axillary meristems, rooting can often be accomplished under ambient environmental conditions directly in commercial soil rooting mixtures. Where this is accomplished, the third and fourth steps can be combined, resulting in a savings of in vitro culture time, growth chamber space, and labor.[3]

[3]Schwarz, O. J. Unpublished

There are several advantages to shoot proliferation via axillary meristems over the *de novo* organogenic route. Clonal plantlets derived from axillary shoots have been reported to be genetically stable, showing no higher frequency of spontaneous mutations than plants derived through seed (Abdullah et al. 1986, Hussey 1986). In addition, the time required for the production of axillary shoots is shorter than that needed for clonal shoots of adventitious origin (Abdullah et al. 1984, Burns et al. 1991, Kolevska-Pletikapic et al. 1983, Zel et al. 1988).

Stimulation of Bud Meristem Development

Stimulation of axillary bud development has been approached in several ways, depending upon the developmental stage of the explant donor. Amerson et al. (1988) successfully produced multiple microshoots from 11-year-old loblolly pines that were severely hedged prior to explant removal. The donor trees were hedged to stimulate the production of shoots possessing "juvenile" morphology with elongating primary needles. Other investigators have used juvenile explant materials, starting with seedlings of various ages up to 3-year-old plants (Table 1).

Explants are disinfested and treated with a cytokinin to stimulate the development of axillary meristems present in the explant tissues. The method of exposure to the cytokinin varies. Generally, explants are obtained from both juvenile and mature sources and incubated on a cytokinin-containing medium for varying periods of time (Abdullah et al. 1986, Horgan 1987, Skripachenko 1982, Toribio and Pardos 1989, Zel et al. 1988). They are subsequently transferred to a hormone-free basal medium to allow the axillary buds to elongate sufficiently for rooting.

Several variations of a pulse exposure method have been described. Zel et al. (1988) obtained maximum axillary bud activation in explants from 9-week-old *P. sylvestris* L. seedlings after a 5-hour soak in an aqueous BA-containing solution. Amerson et al. (1988) pulse-treated explants from 11-year-old clonal loblolly pines for 1 to 2 minutes by immersion in a 70% ethanol solution containing BA. A slight modification of this method was successfully applied to hypocotylary explants of 5-week-old slash pine seedlings (Burns et al. 1991). The slash pine seedling explants were pulse-treated in an aqueous 70% ethanol:0.1% dimethyl sulfoxide (DMSO) solution containing BA for 45 seconds. This shortened exposure time was sufficient to produce an average of three to seven harvestable axillary shoots per genotype, depending upon seed source. The concentration of BA used to treat the various types of explants varied with maturation state and method of hormone exposure. In general, the more juvenile the explant, the lower the cytokinin concentration required to stimulate axillary bud development.

An in vitro methodology for the spontaneous production of axillary shoots without exogenous hormone application has been described for several *Pinus* species by Baxter et al. (1989). Axillary shoot production depends solely upon the repeated topping or hedging of elongating seedling shoots and the repeated harvesting and elongation of the resulting axillary shoots. A high level of interclonal variation was found with respect to axillary shoot production. The interclonal variability with respect to axillary shoot production also varied between species under constant cultural conditions. Greater than 1000 explants were produced in 1 year by 11.8% of the *Pinus oocarpa* clones compared to *Pinus caribaea* at 2.5%, and *Pinus tecunumanii* at 0%.

Elongation of Axillary Shoots

After activation of the quiescent meristems by the cytokinin treatment, explants were usually transferred to basal medium with a decreased cytokinin concentration (Abdullah et al. 1987) or to basal medium without hormones to promote shoot growth (Abdullah et al. 1986, Burns et al. 1991, David 1982, Horgan 1987). This shoot elongation phase may require only a single transfer to achieve an appropriate shoot size for subsequent rooting (Amerson et al. 1988, Burns et al. 1991). The methodology used to promote axillary shoot elongation is essentially the same as that described for shoots of adventive origin (see discussion above).

ADVENTITIOUS ROOTING AND ACCLIMATION

Adventitious Rooting

To complete plantlet regeneration, elongated shoots obtained from either adventive or axillary meristems were placed under various regimes designed to induce adventitious root meristem formation. Mohammed and Vidaver (1988) reviewed the literature concerning the procedures for rooting and acclimation of micropropagated conifers. These authors provided an extensive tabulation of rooting

treatments that have been used with conifer tissue cultures. Adventitious rooting was found to be influenced by the method of application and concentration of the rooting hormone, physiological state of the shoot ("shoot quality"), age of the original explant donor, donor genotype, and temperature. In addition, the morphogenic origin of the microshoot, i.e., shoots of adventive organogenic origin as opposed to pre-existing axillary buds, also affects rooting success[4] and the subsequent field performance of the plantlets (Abdullah et al. 1989).

A review of the literature describing rooting protocols indicates that a general trend has developed for in vitro adventitious root induction. The process involves the exposure of the basal portion of the microshoot to the inducing hormone. Root-inducing protocols include a relatively short duration pulse (Aitken-Christie and Thorpe 1984, Bornman 1983, Chesick et al. 1991, Patel and Thorpe 1986), an extended hormonal exposure (Bronson and Dixon 1991, Chesick et al. 1991, Coleman and Thorpe 1977, Mohammed et al. 1989), a dip in rooting powder (Patel and Thorpe 1986), or simply maintaining the shoots on basal media without hormones to allow spontaneous rooting (Burns et al. 1991, David 1982, Horgan and Aitken 1981, Webb et al. 1988). The efficiency of adventitious rooting is highly variable and remains one of the key problems encountered in conifer plantlet regeneration in vitro (Burkhart and Meyer 1991, Haissig et al. 1992, Mohammed and Vidaver 1988). It has been found that rooting occurs when tissues are exposed for a specified time to an auxin (in the form of indole-3-acetic acid [IAA], naphthalene acetic acid [NAA], indole-3-butyric acid [IBA]), alone or in combination with a cytokinin (e.g., BA). The microshoot is then transferred to a hormone-free basal medium to promote the further development and elongation of newly initiated root meristems. Usually the concentration of mineral nutrients in the basal medium is reduced to one-half to one-fourth that used for shoot initiation. The soluble sugar (sucrose, maltose, etc.) level varies but is usually set at 1 to 2%. Photoperiods from 12 to 24 hours and both reduced and elevated temperatures, when compared with those used for shoot induction and elongation, have been used in rooting protocols. The parameters of rooting protocols are highly dependent on an array of culture variables and environmental conditions that must be adjusted to the specific genotypes to be rooted. The presence or absence of charcoal in the agar rooting medium (Mohammed and Vidaver 1988), the use of gibberellin synthesis inhibitors in combination with auxin (Burkhart and Meyer 1991), the substitution of peat-perlite for agar as the support medium, and the addition of boron to the induction and elongation medium (Mohammed et al. 1989) are all examples of culture variables shown to affect upon rooting efficiency. For a detailed discussion of these and other related culture variables, see Mohammed and Vidaver (1988) and Thorpe et al. (1991).

Plantlet Acclimation

Problems associated with the acclimation of conifer plantlets to ambient environmental conditions can be minimized if rooting is carried out under *ex vitro* conditions directly to soil in the greenhouse. Under these circumstances, both rooting and acclimation are accomplished in a single step. Experience with several conifer species (e.g., *Cupressus lusitanica*, *Pinus oocarpa*, *Pinus elliottii*) has encouraged further development of this combined approach.[5] Of greatest concern when transferring in vitro-generated plantlets to the greenhouse and eventually to field conditions is excessive water loss. This is usually related to the absence or greatly reduced levels of epicuticular wax on the leaf (i.e., needle) surfaces (Sutter 1982). Early methods of transitioning usually involved placing the regenerant in a pot of soil covered with a container or beaker located in the laboratory to facilitate its close observation. The plantlet was carefully watered to maintain a condition of high humidity without saturating the soil. After an initial period, usually ranging from 10 to 14 days, the container/beaker was gradually raised to reduce humidity. At the end of an additional 14 days, the container/beaker was completely removed, and the plantlet was ready for transfer to the greenhouse. This level of attention is not possible when large numbers of plantlets are generated. The availability of humidity control systems for greenhouses has enabled acclimation of in vitro-rooted plantlets and the single-step, direct-to-soil rooting of microshoots. By no means has the problem of low rooting efficiency and plantlet mortality during acclimation been solved; however, greenhouse environmental control technology has provided an added tool with which to help solve the problem.

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[4]Schwarz, O.J., unpublished.

[5]Schwarz, O.J., unpublished.

PERSPECTIVES AND CHALLENGES

Many challenges and limitations need to be overcome before the mass propagation of forest trees via in vitro methods can become routine practice. All of the micropropagation technologies reviewed in this paper are still in their infancy, in particular, with reference to cloning efficiency and, more importantly, with respect to understanding of the primary biological mechanisms controlling the processes of differentiation and growth. The disciplines of genetics, plant physiology, biochemistry, and molecular biology will be required to gather basic knowledge on nutrition, hormonal action, and the control of the expression of totipotency in cells of both juvenile and mature tissues (Haissig 1989, Haissig et al. 1987, Haissig et al. 1992, Hussey 1986). Significant accomplishments have been made in regeneration in vitro when the explant is derived from seed or other juvenile tissues. However, successful application of current micropropagation technologies to a mature tree that has been identified as genetically superior is not routinely accomplished. The reason for limited success is that selection can only be reliably made at a time when the individual is morphologically/ developmentally mature and, in comparison to embryonic tissue or seedlings, has, to a great degree, lost its morphogenetic plasticity (Bonga 1987, Mason and Gill 1986). Ununger and Ekberg (1987) believe that "The most serious constraint associated with clonal forestry is the physiological changes within the tree that are related to its aging." Accordingly, the use of cuttings for propagation of *Picea abies* (Ununger and Ekberg 1987) and other conifer species (Mason and Gill 1986) in commercial ventures has been limited to trees of 4 to 6 years of age.

Difficulties similar to those associated with the rooting of coniferous species from cuttings of mature trees are encountered in every stage of micropropagation technology. Figure 1 illustrates the developmental progression towards maturation of a typical coniferous forest tree, together with representative explant materials that have produced developing microshoots and, in the case of the juvenile materials, have routinely regenerated plantlets and emblings. There have been numerous approaches to increasing the micropropagation efficiency of more mature, less juvenile explanted tissues of both hardwood and coniferous forest trees (Franclet et al. 1987). In general, the methods outlined rely on two approaches: (1) the utilization of those portions of the mature tree thought to be juvenile by virtue of their placement within the tree or the timing of their initial morphogenesis (Ballester et al. 1990, Durzan 1990) and (2) rejuvenation of selected tissues and organs through cultural manipulation, chemical treatment, or a combination of both (Bonga 1987, Thorpe and Harry 1990). In some instances, published research has relied on an empirical approach to experimental design, based mainly upon previous successes. Major advances are likely to emerge when the existing body of empirical knowledge can be combined with an increased understanding of the basic mechanisms that control the transition from juvenility to maturity.

In consideration of the three major in vitro propagation pathways discussed in this review, the most promising in the long term appears to be somatic embryogenesis. This propagation route offers the greatest potential for large scale commercial application, while providing the ideal system for genetic engineering. Unfortunately, this system is the least developed with respect to its efficacy and species applicability. In the interim, the opportunity to use clones of selected trees provided via organogenic or axillary bud propagation in existing tree improvement programs should be vigorously pursued (Zobel 1992).

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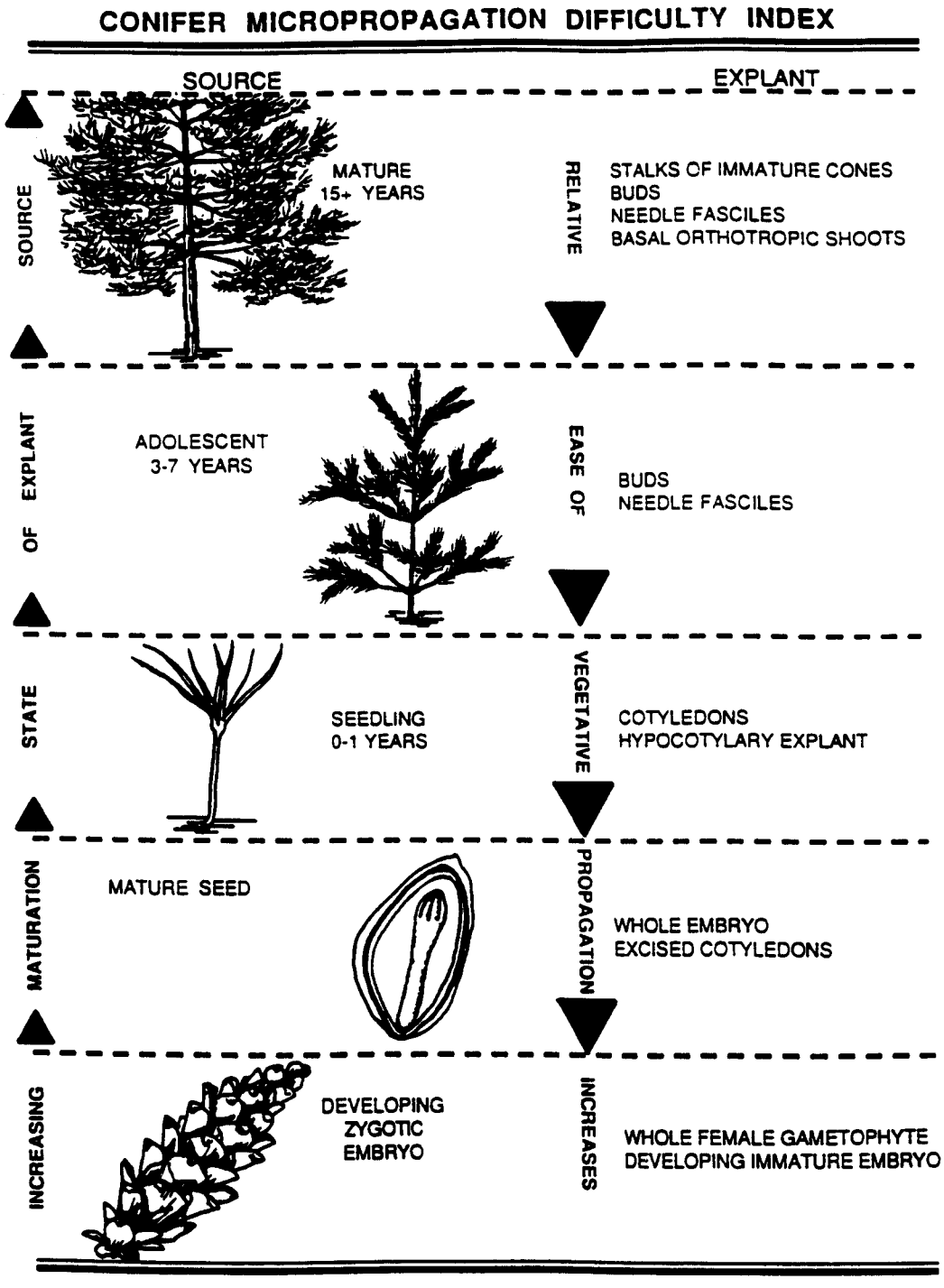

Figure 1. The developmental stages in the life of a typical conifer as they relate to examples of increasingly mature explant tissues that have been used for micropropagation. For most conifer forest trees, the ease of vegetative propagation, whether by traditional or in vitro methods, decreases with increasing maturation of the donor tree.

IN VITRO PROPAGATION OF HARDWOODS[1]

S.A. Merkle and R.N. Trigiano[2]

Abstract.--Despite substantial advances in propagation of hardwood forest trees during the last decade with regard to the number of species successfully regenerated and frequency of propagules produced, there are still relatively few systems for southern hardwood species that are useful for operational purposes. Shoot or bud multiplication systems, while regarded as producing the most true-to-type-propagules, are labor-intensive and multiplication rates are relatively low. Organogenic systems, while offering the potential for higher-frequency propagule production, still require multiple labor-intensive steps for production of plantlets ready for field planting, and may be subject to higher amounts of within-clone variation due to the origin of the shoot meristems. Embryogenic systems appear to offer the highest potential for large-scale propagule production at a low per unit cost, by high multiplication rates and by eliminating the multiple steps required by the other systems. However, very few embryogenic systems have been initiated from mature hardwood trees, limiting propagation of plants of proven genetic value. The combination of different in vitro regeneration systems (and conventional propagation systems) may provide the best potential for producing material from superior genotypes on an operational scale. Examples of current in vitro propagation technology with southern hardwoods include shoot multiplication and organogenic systems for sweetgum (*Liquidambar styraciflua*), organogenic regeneration systems for eastern cottonwood (*Populus deltoides*) and embryogenic regeneration systems for yellow-poplar (*Liriodendron tulipifera*), black locust (*Robinia pseudoacacia*) and American chestnut (*Castanea dentata*).

INTRODUCTION

Historical Perspective and Current Effort

In vitro regeneration systems offer a number of potential applications for mass propagation of superior genotypes of hardwood forest tree species. Hardwood forest trees were among the first plants cultured in vitro. Gautheret (1940) reported formation of adventitious buds in cultured cambial explants of smooth-leaved elm (*Ulmus campestris*), demonstrating that an exogenous source of sugar was required for bud production, while high levels of indoleacetic acid (IAA) in the medium inhibited bud formation. Jacquiot (1949) extended the research with this species, using trees up to 180 years old as tissue sources. Mathes (1964) reported in vitro production of both roots and shoots in callus cultures of quaking aspen (*Populus tremuloides*), although apparently no plantlets were produced. It was not until four years later that Wolter (1968) produced entire *P. tremuloides* plantlets in vitro by inducing shoot formation with benzylaminopurine (BAP) and subsequently rooting the shoots in vitro. Winton (1968), working with triploid *P. tremuloides* callus, also obtained complete plantlets, demonstrating for

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the first time in vitro propagation of a forest tree with a superior genotype.

Since the pioneering work with *Populus*, hundreds of hardwood tree species have been propagated in vitro, although much of this work was done only on an experimental rather than an operational scale. In the United States, during the last decade, research priority was placed on commercially important coniferous species. However, a number of laboratories have contributed to substantial progress in the field of hardwood tissue culture, resulting in the availability of a few tissue culture regeneration systems that are ready to be employed for applied purposes. It would be unrealistic to attempt to review all of the tissue culture research reported to date with North American hardwoods. However, it is also true that, whereas a number of comprehensive reviews of in vitro propagation of coniferous trees are available, the same cannot be said for North American hardwood trees. Fortunately, there are a number of books which, between them, contain chapters on the in vitro propagation of virtually all of the important hardwood genera represented in North America. We would refer those looking for information on manipulation of individual hardwood genera in vitro to books edited by Bajaj (1986, 1989, 1991) or Bonga and Durzan (1987).

Types of In vitro Propagation Systems

Systems for propagation of plants from sterile culture, also known as micropropagation, fall into three broad categories, one of which relies on multiplication of preformed structures, and two of which rely on *de novo* generation of either plant organs or embryos (i.e. morphogenesis). These three systems are axillary shoot or bud multiplication (a.k.a. axillary enhancement), organogenesis and somatic embryogenesis.

Production of plantlets from axillary shoots is similar to vegetative propagation via rooted cuttings, in that shoots are excised from source tissue and rooted individually to multiply the original genotype. The differences are that, with axillary shoot systems, the procedures are performed in vitro, under aseptic conditions, and that manipulations in vitro can be used to greatly multiply the number of propagules obtained. These include media supplements and culture conditions which induce axillary buds in the cultured tissues to elongate to form shoots. These shoots can then be excised and either rooted in vitro, or placed back in culture for further enhancement of axillary branching. Thus, unlike rooted cutting macropropagation, conditions can be applied in vitro which promote multiplication of new shoot material.

Organogenesis is the *de novo* production of plant organs (buds, shoots, roots) from tissues or from callus. In many cases, initial explants are induced to form callus and subsequently shoots. Then, similar to axillary shoot methods, the shoots are induced to elongate, are excised and rooted individually. Somatic embryogenesis is the *de novo* production of structures resembling zygotic embryos, either from organized tissues or from callus. Structures classified as somatic embryos must be bipolar (possessing both root and shoot poles) and have no vascular connection to the source tissue. Somatic embryos may be derived either through direct or indirect embryogenesis. In direct embryogenesis, embryos are formed essentially by multiplication of a zygotic embryo explant, i.e by embryo cloning (Sharp et al. 1980). Indirect embryogenic systems involve a dedifferentiation of nonembryonic tissue to form a callus from which somatic embryos arise (Sharp et al. 1980). These two forms of somatic embryogenesis have an impact on the potential application of embryogenic systems for propagation and will be discussed further.

In vitro propagation methods covering all three routes of regeneration have been widely reported for hardwood forest trees. However, even after years of experience, only a few methods are being applied or have a real potential to be applied for operational production of hardwood forest tree propagules. Our purposes of this review, therefore, are to (1) present a view of the goals of in vitro production of hardwoods, with regard to operational applications, (2) discuss features which impact the operational utility of in vitro propagation methods, (3) Compare different in vitro propagation methods with regard to their relative advantages and disadvantages for operational application to hardwood species, (4) present a number of examples of in vitro propagation systems for hardwood species that demonstrate the current state of the art for hardwood micropropagation, and (5) briefly speculate about some advances in methodology which are likely to have some impact on application of this technology to hardwood forest trees in the future. We have chosen to limit the scope of the examples in two ways: We will concentrate mainly on southern hardwood species, although a few

systems for other hardwoods will be cited, and we will focus on describing those systems which are state-of-the-art for in vitro technology for these species.

GOALS AND DESIRABLE FEATURES OF IN VITRO PROPAGATION SYSTEMS

Goals of In vitro Propagation of Hardwoods

A primary goal of vegetative propagation is to capture the total genetic superiority of the parent material, which includes both additive and nonadditive genetic components. In addition, clonal propagation systems allow the application of a very high selection differential, since whole new populations of plants can be cloned from just a few elite individuals. Theoretically, since somatic tissues are used as the starting material, trees regenerated from these tissues should be exact clonal copies of the ortet. However, unlike macropropagation systems, some in vitro propagation methods have the potential to introduce significant new genetic or epigenetic changes (somaclonal variation) into propagules regenerated from them, so that the parent material is not truly replicated. Furthermore, the nature of much in vitro propagation technology for hardwood species excludes clonal propagation of proven genotypes, since many of these systems are limited to employing explants from juvenile, genetically unproven material.

A second principal goal of micropropagation that parallels conventional vegetative propagation is simply to mass propagate plants, bypassing sexual reproduction, which for some reason may be expensive, inefficient or impractical. However, in vitro propagation can also be used to overcome problems associated with macropropagation, including limitations on available parent material or space. In addition, just as propagation through seeds may be limited by biological factors, certain species or even individual genotypes may be recalcitrant to conventional vegetative propagation techniques, such as rooting of stem cuttings. In these cases, in vitro techniques may succeed where macropropagation fails.

In addition to the advantages shared with conventional vegetative propagation, in vitro culture has a number of associated applications that are unique. For example screening of genotypes for such traits as disease resistance can be accomplished in vitro, followed by production of propagules from the selected material. This technique has already been applied with some coniferous trees (Amerson and Mott 1990). In vitro selection has also been used in conjunction with somaclonal variation to generate new variants in culture, which can then be propagated in vitro. This technique has been used to generate hybrid *Populus* variants that are resistant to the herbicide glyphosate (Michler et al. 1992) and eastern cottonwood (*Populus deltoides*) variants that show increased or decreased resistance to leaf rust caused by *Melampsora medusae* (Prakash and Thielges 1989).

Another potential application of in vitro culture which may eventually succeed for hardwood trees is generation of somatic hybrids via protoplast fusion. For example, protoplasts of *Citrus* species from different genera which were unable to hybridize sexually, were fused and somatic hybrid plantlets regenerated (Grosser et al. 1988). Although regeneration of somatic hybrids has not been reported for a hardwood forest tree to date, protoplasts of a number of species have been isolated and induced to regenerate cell walls and divide to eventually produce protoplast-derived plantlets (e.g. Russell and McCown 1986, Sticklen and Lineberger 1985, Merkle and Sommer 1987). Finally, in vitro culture provides the only route for generation of genetically engineered genotypes of hardwood trees. Gene transfer technologies including *Agrobacterium*-Ti plasmid mediated gene transfer, electroporation, microinjection and microprojectile bombardment all depend on the ability to culture cells in vitro in order to select the transformed cells, usually by employing drug-resistance marker genes. Once transformed cells are obtained, these can be cultured to regenerate transformed plantlets. It is of special value to be able to employ cultures capable of regeneration from single cells, since this ability raises the probability that transformants derived from these cultures will be nonchimeric.

Desirable Features for In vitro Hardwood Propagation Systems

The ability to regenerate a particular hardwood species from tissue culture does not necessarily guarantee that the method will be useful for operational propagation of that species. To be adapted for production purposes, an in vitro propagation system must have a number of attributes that make it an economically feasible alternative to conventional techniques. A number of these attributes are determined by the type of in

vitro propagation system (i.e. shoot multiplication, organogenesis or somatic embryogenesis).

A primary consideration for propagation purposes is the frequency with which plantable propagules are produced. Due to the high labor inputs for initiating and maintaining plant tissue cultures and producing field plantable stock from them, costs are high compared to conventional systems. Thus, in order to compete with macropropagation methods on a per unit cost basis, micropropagation systems must possess the potential to produce thousands of trees from a single explant in a limited time. Therefore, an in vitro propagation system must be amenable to scale-up, in order to lower the cost per propagule. Additional savings in labor costs may be obtained if the system can be automated. A very important factor with regard to the successful application of an in vitro propagation system is the ability of the cultures to generate plantlets that are true-to-type, i.e. that are faithful to the phenotype of the parent material. Since one of the theoretical advantages of clonal propagation is that members of the clone will be exact copies of the desirable ortet, one of the primary motivations for clonal propagation is lost if the regenerants vary significantly from the phenotype of the ortet. Also, when in vitro cultures are started from immature, genetically unproven material, which is the case for many hardwood tree cultures, clonal uniformity is still a desirable feature for the regenerants. Thus, in vitro propagation methods with a high potential for somaclonal variation are less desirable for clonal production than those that can guarantee propagules with uniform performance.

Finally, since even very efficient in vitro propagation methods are still more expensive on a per unit cost basis than seedlings for most species, micropropagation methods must be able to offer some value-added component to the regenerated trees in order to make the system economically feasible (Redenbaugh et al. 1987). A value-added feature is a phenotypic character that in vitro regenerated propagules possess that is unavailable in trees regenerated by other means, which makes up for the added cost of the culture-derived trees. One example of such a value-added feature is genetically engineered genotypes, carrying and expressing foreign genes for such traits as insect or disease resistance. Another such feature may be the production of rare hybrid trees that cannot be achieved in large numbers in a reasonable time period through conventional breeding.

ADVANTAGES AND DISADVANTAGES OF DIFFERENT IN VITRO PROPAGATION SYSTEMS

After a number of years of experience with the three in vitro regeneration methods with a variety of hardwood forest tree species, there is some basis for comparison of the different methods. Using the criteria listed in the previous section, these methods are compared with regard to their relative advantages and disadvantages for application to propagation of southern hardwood trees. However, as the examples will show, the success of a given method appears to be highly species-specific, with shoot multiplication systems working well for some species, while embryogenic systems are clearly superior for others. We choose to emphasize here that it is counterproductive to view these different systems as competing. Indeed, it may ultimately be more beneficial to combine the strongest features of the different systems in order to achieve the desired goal, or even to combine in vitro propagation systems with conventional vegetative propagation systems, which has been accomplished in one of our later examples.

Axillary Shoot Multiplication

Probably the main advantage of axillary shoot multiplication methods for hardwood trees is that since all propagules are derived from preformed buds, this method provides the best guarantee that propagules will be true-to-type. Since shoots arise from meristems present in the explant, there is little chance of introducing somaclonal variation into the propagules. Currently, axillary shoot multiplication methods offer the best (and sometimes only) route for propagating mature genotypes. Mature genotypes of such species as black locust (*Robinia pseudoacacia*) have been propagated via axillary shoots, while organogenic and embryogenic systems for these species have relied on immature starting material.

Given that production of plants from axillary shoots has some similarities to rooted cutting propagation, it might be expected that it has a number of disadvantages in common with this macropropagation technique. One of these is the relatively low frequency of propagule production associated with this technique. Although repeated cycles of in vitro culture can ultimately produce thousands of propagules, large input of labor and facilities are required to produce operationally useful numbers of plants. Axillary shoot

multiplication methods are also relatively labor-intensive, requiring substantial handling, both for cycling of cultures and for production of plantlets. Plantlet production can require multiple steps, including induction, shoot elongation, shoot excision, rooting and acclimatization of plantlets, each of which may require significant input of labor. Finally, because of multiple handling steps, axillary shoot multiplication methods may not be as amenable to scale-up or automation as other methods for hardwood forest tree propagation, although recent advances in robotics may change this potential.

Organogenesis

The primary advantage of organogenic regeneration methods over axillary shoot methods is that some of these methods may have the potential for higher frequency plantlet production in a shorter period of time than axillary shoot systems. Callus can be grown in large quantities with less demand for inputs of labor and space, and adventitious shoot production can be used to achieve high multiplication rates. However, like axillary shoot methods, organogenesis-based methods may require the labor-intensive steps of shoot elongation, excision, rooting, and plantlet acclimatization to produce field-plantable stock. In addition, unlike axillary shoot methods, organogenic methods, especially those requiring an intermediate callus, are associated with the production of significant amounts of somaclonal variation in the regenerated plantlets. Thus, the resulting plantlets from a given clone may display unacceptable variation.

Somatic Embryogenesis

Somatic embryogenesis has been cited by many authors as the in vitro regeneration system of choice for economical production of clonal populations of forest trees (e.g. Gupta et al. 1991). Certainly, this type of system has a number of powerful advantages over axillary shoot multiplication methods and organogenesis. However, it should be noted that the efficiency of embryogenic methods varies widely with species. One important advantage of embryogenic methods over both axillary shoot and organogenic methods is the potential for very high frequency regeneration. Depending on the species, virtually unlimited numbers of embryos can be generated from a single explant. In addition, embryogenic cultures of many species can be grown in liquid, allowing production and handling of thousands of embryos at one time. Thus, in comparison to axillary shoots and organogenesis, somatic embryogenesis offers the potential for high volume, large-scale propagation which can be translated into significant labor savings. Greater economies of scale may be possible if bioreactor and continuous culture technologies can be applied to embryogenic systems (e.g. Styer 1987, Stuart et al. 1987). The feature of embryogenic methods which may ultimately have the most impact for mass propagation of hardwood trees is the fact that the product is an embryo. The morphological and physiological similarity of somatic embryos to zygotic embryos means that they are complete propagules in themselves, with embryonic roots, shoots and leaves, and, most importantly, the "program" to make a complete plant. Thus, unlike other in vitro propagation methods, no separate shoot elongation or rooting steps are required for plantlet production. Furthermore, many embryogenic systems produce singularized embryos, requiring no excision from source tissue or other embryos in order to be handled. These characteristics further lower labor inputs and give somatic embryos the potential for direct delivery to the greenhouse or field as "artificial seeds" (Redenbaugh et al. 1987, Fujii et al. 1992), thereby eliminating the need for labor-intensive transplanting.

A major disadvantage of embryogenic propagation methods for hardwood species is that the bulk of forest tree embryogenic systems reported to date have relied on immature tissues (i.e. from seeds or seedlings) as explanting material. Thus, the material being propagated is of unproven genetic value. Most reports of somatic embryogenesis in tree species are in reality reports of "embryo cloning," in which the zygotic embryo is induced to replicate itself indefinitely. Another drawback of forest tree embryogenic systems concerns the low frequency of plantlet production or "conversion" of somatic embryos to plantlets. Although numerous embryogenic systems have been reported for forest trees, some of which produce embryos at high frequencies, a major bottleneck has been induction of embryo maturation and subsequent production of field-plantable stock. Currently only a few species of North American hardwoods can be propagated via somatic embryogenesis from multiple clonal lines in sufficient numbers to enable establishment of useful field tests.

Finally, the relative advantages or disadvantages of somatic embryogenesis for propagation of a given species depend on whether the system developed is direct or indirect. Direct embryogenic systems,

where there is no intermediate callus stage have been cited as having a very low potential for the production of variants (Ozias-Akins and Vasil 1988, Maheswaran and Williams 1987). Thus plantlets produced via this route should be very faithful to their clone. However, embryos produced via direct systems tend to proliferate as clumps of mixtures of globular and other stage embryos. Thus, they may not grow well in suspension cultures and will require more handling to produce individual plantlets, raising labor costs. Conversely, indirect embryogenesis, in which embryos are produced from callus, has a higher potential for maintenance in suspension culture and for production of singularized embryos, which may be synchronized in development for ease of handling. However, the callus origin may give indirect embryogenic cultures a higher potential to introduce unwanted variation into the clonal population (Maheswaran and Williams 1987).

ILLUSTRATIONS OF HARDWOOD IN VITRO PROPAGATION SYSTEMS

The following examples demonstrate that the three in vitro propagation methods have the potential to be adapted for operational use with some hardwood species. Here, we have concentrated on choosing systems with southern hardwood species as examples, with the inclusion of a few other hardwoods that are good illustrations of what can be accomplished with a particular species or method.

Sweetgum

There are more published reports on in vitro propagation of sweetgum (*Liquidambar styraciflua)* than any other major southern hardwood tree, either via axillary shoot multiplication (Sutter and Barker 1985), adventitious shoot formation (Sommer 1981, Sommer et al. 1985, Brand and Lineberger 1988, 1991, Chen and Stomp 1991) or somatic embryogenesis (Sommer and Brown 1980). Here, three systems are summarized, one of which is currently being applied for operational production of sweetgum clonal material.

Sommer et al. (1985) described an organogenic regeneration system for sweetgum which was capable of producing up to 50 plantlets per culture on approximately a monthly basis, depending on clonal line. To initiate the cultures, seedlings were germinated aseptically and hypocotyl sections were explanted on a semisolid Risser and White's (1964) medium with 0.1 mg/l IAA and 0.5 mg/l BAP to initiate adventitious shoot production. For shoot multiplication, Sommer et al. (1985) found that a liquid Blaydes' (Witham et al. 1971) medium with 0.01 mg/l naphthaleneacetic acid (NAA) and 0.5 mg/l BAP was superior to an agar-solidified medium (Sommer 1981). Adventitious shoots were rooted with up to 90% efficiency on a modified Risser and White's (1964) basal medium. Sommer et al. (1985) also reported on nursery bed performance of the plantlets derived from adventitious shoots, noting some problems with root girdling.

Brand and Lineberger (1988, 1991) also reported on an organogenic regeneration system for sweetgum, in which adventitious shoots were induced to form directly on leaf explants, without formation of callus. In the first study, leaf and petiole tissues were harvested from shoot cultures initiated from lateral buds of 25- to 30-year-old trees and explanted onto a Woody Plant Medium (WPM; Lloyd and McCown 1980) with 2.5 mg/l BAP. Shoot formation on leaf tissues occurred most frequently at or near breaks in major vasculature. Wounding the leaves by cutting across the lamina and vasculature increased the number of shoots formed per explant up to 18 for one sweetgum variety. Over 88% rooting of the excised shoots was obtained with a 2-min basal dip in 200 mg/l IBA prior to transfer to a peat/vermiculite mixture in covered trays. Later, Brand and Lineberger (1991) applied the same shoot induction treatment to obtain up to 57 shoot meristems per explant when leaves from intact plants were used instead of leaves from shoot cultures. Leaf developmental stage exerted an impact on shoot meristem production, since leaves that were 50% expanded produced up to 90 shoot meristems per explant for one sweetgum variety.

Workers at Union Camp Corporation have applied a sweetgum shoot multiplication method to propagate selected genotypes to be used in operational production of sweetgum. The method, outlined briefly by Feirer and Wann (1989), and modified from that of Sutter and Barker (1985), depends on shoot multiplication from apical meristems of selected trees up to several years old. Dormant buds or greenwood cuttings were sterilized by application of both mercuric chloride and bleach. Excised shoot tips were placed on WPM with 0.01 mg/l NAA and 2 mg/l BAP. Shoot cultures developed mainly from axillary buds, and were maintained on the same medium. Shoots were given a rooting

pretreatment in vitro, by placing excised shoots on 1/3-strength WPM with 0.1 mg/l indolebutyric acid (IBA) and 1% sucrose for 3 weeks. Pretreated shoots were moved to peat plugs for root emergence under fog. Over 100 mature trees selected from genetic tests and seed orchards have been propagated using this method (personal communication, S.R. Wann, Union Camp Corp., Princeton NJ). To date, over 6000 plantlets have been produced from over 60 clones, with no obvious phenotypic variants. The method is in use in Union Camp's vegetative propagation program for "clone capture," i.e. to make sufficient copies of a selected tree for planting in a cutting orchard. Union Camp's sweetgum program is an excellent example of a combination of vegetative propagation methods (i.e. micropropagation and rooted cuttings) for a specific application, and, to our knowledge, represents the only application of in vitro propagation to operational production of selected genotypes of a southern hardwood.

Eastern Cottonwood

More cell and tissue culture research has been reported on *Populus* than for any other hardwood genus. For the purposes of this review, we will focus attention on eastern cottonwood (*Populus deltoides*), which is well known for its capacity to be highly productive in the southeastern United States. For further information on *Populus* tissue culture, we refer the reader to reviews by Ahuja (1987), and papers on protoplast culture and somatic embryogenesis by Russell and McCown (1986) and Michler and Bauer (1991), respectively.

Coleman and Ernst (1989, 1990, 1991) conducted the most intensive research program with organogenic regeneration of eastern cottonwood, although their research concentrated more on the control of adventitious shoot regeneration than on optimizing plantlet production. In the 1989 study internodal explants from 3 clones collected from a stool bed were explanted onto modified WPM with various concentrations of the cytokinins BAP, zeatin and 2-isopentenyladenine (2iP). Zeatin proved to be the best cytokinin for adventitious shoot production, while BAP was apparently phytotoxic. A zeatin concentration-by-genotype interaction was also observed. Further investigations (Coleman and Ernst 1989) revealed that when zeatin concentration was held at 0.5 mg/l, there were strong genotypic differences in shoot regeneration among 16 genotypes. Coleman and Ernst (1990) further defined shoot regeneration competence and callus determination in eastern cottonwood in terms of developmental stages, finding some genotypes that were competent for direct shoot regeneration, other genotypes requiring initial culture on callus-inducing medium with 0.5 mg/l 2,4-dichlorophenoxyacetic acid (2,4-D) to acquire shoot regeneration competence, and some genotypes which would never regenerate shoots. Later, Coleman and Ernst (1991), using SDS-PAGE and 2-D electrophoresis, showed that certain proteins were associated with explants determined for either shoot regeneration versus callus growth.

Adventitious shoot regeneration was also reported for eastern cottonwood using leaf disk explants (Prakash and Thielges 1989, Uddin and Dinus 1990), although regeneration has been limited to a few genotypes. Prakash and Thielges (1989) cultured leaf disks on modified WPM with 1 μM NAA and 1 μM BAP to obtain callus from the cut edges of the explants. Callus was subsequently transferred to modified WPM with 0.1 μM thidiazuron (TDZ) for production of adventitious shoots. Shoots were elongated on WPM with 0.5 μM BAP, excised, and grown on WPM with 0.1 μM BAP prior to rooting on peat plugs. When the resulting somaclones were screened for resistance to *Melampsora* leaf rust, some individuals displayed increased resistance or susceptibility. Uddin and Dinus (1990) also reported adventitious shoot production from eastern cottonwood leaf sections cultured on WPM supplemented with NAA and BAP, which were elongated by reducing growth regulator concentrations to half-strength. Excised shoots could be rooted on WPM supplemented with IBA. The authors proposed to use this regeneration system as the basis for *Agrobacterium*-Ti plasmid-mediated gene transfer.

Another development involving in vitro technology for eastern cottonwood should be noted. Bradshaw et al. (1992) are constructing a restriction fragment length polymorphism (RFLP) map in a *Populus* pedigree founded by a black cottonwood (*P. trichocarpa*) female and a *P. deltoides* male. Stem explants of the *P. trichocarpa* parent on zeatin-supplemented WPM produce adventitious shoots with 100% frequency, while those from the *P. deltoides* parent fail to produce adventitious shoots. In the F_2 and backcross, continuous segregating variation from 0-100% is found. By correlating adventitious shoot formation in vitro with RFLP genotypes, Bradshaw et al. (1992) intend to elucidate the genetics of organogenesis in the genus.

Silver Maple

Although silver maple (*Acer saccharinum*) is not generally considered to be a major southern hardwood, recent progress in micropropagation of this species may have implications for propagation of other hardwoods, especially with regard to the application of the relatively unknown plant growth regulator, TDZ. Preece et al. (1991a,b) cultured 2.5 cm shoot tips or nodal explants from juvenile and mature trees on a Driver and Kuniyuki (1984) medium supplemented with different concentrations of four amino purine cytokinins (BAP, kinetin, zeatin and 2iP) and TDZ, a substituted phenylurea compound. TDZ at 10 nM was found to be optimum for production of large numbers (over 100 after 4 months) of long shoots by both juvenile and adult nodal explants. The authors believed that in addition to long-term exposure to TDZ, the high frequency shoot production could be attributed to decapitation of the longest shoots in the mass to release apical dominance and the development of a massive callus base, which provided physical support for the shoots in liquid culture and may have aided with nutrient uptake from the medium. Microshoots of juvenile origin which were 1.5 cm or longer could be rooted at a high frequency in the greenhouse under intermittent mist, with no auxin treatment, while rooting of microshoots of adult origin apparently was improved by a 15-second dip in 1 Mm IBA. Preece et al. (1991b) concluded that their micropropagation method could be applied successfully to a wide range of silver maple genotypes. To date hundreds of micropropagated trees have planted in the field and have performed comparably with bare-root seedlings (Preece et al. 1991a).

Black Locust and Other Woody Legumes

While there are a number of woody leguminous species in North America, the one with substantial commercial potential as a forest species is black locust (*Robinia pseudoacacia*). However, results with in vitro propagation of black locust and other North American woody legumes may have considerable value if it can be applied to other members of the group, since woody legumes are of great importance as timber species in other parts of the world, especially in the dry tropics.

Black locust, while not particularly associated with the southern United States, is of particular interest in this review because it has been regenerated in vitro by axillary shoot multiplication, organogenesis and somatic embryogenesis, and the relative merits of the three regeneration methods can be compared for the same species. It should also be noted that black locust micropropagation has been accomplished from both immature and mature tissues. Chalupa (1983) described an axillary shoot system initiated from nodal segments of one- to three-year-old seedlings. Explants were cultured on a Murashige and Skoog's (1962) medium (MS) supplemented with 0.4-0.6 mg/l BAP and 0.05 mg/l IBA. Shoots were multiplied by excising axillary shoots and culturing nodal segments from them on fresh medium. Shoots were rooted on a modified Gresshoff and Doy's (1972) medium (GD) with 0.3 mg/l NAA and 0.3 mg/l IBA. Similarly, Barghchi (1987) employed axillary bud explants from 2-year old cutting-derived plants cultured on MS medium with 0.25-1.0 mg/l BAP to obtain 5-fold shoot multiplication every month. Shoots were rooted on half-strength MS medium with 1 mg/l IBA. In both reports, plantlets were readily established in soil. Davis and Keathley (1987) induced axillary shoot production from 20- to 30-year-old black locust trees by culturing excised dormant winter buds on MS medium supplemented with 3.2 μM BAP. Shoots were elongated on MS medium with 0.3 μM BAP and rooted on 1/10 strength MS medium with 0.2 mg/l IBA. Adventitious shoot production was also induced in black locust using callus derived from seedling hypocotyl explants cultured on MS medium with 10 μM BAP (Han and Keathley 1989). Shoots derived from the callus cultures and transferred to MS medium with 0.32 μM BAP produced an average of 5 new shoots per culture in 4 weeks.

Embryogenic cultures of black locust were initiated at a low frequency by culturing immature seeds (2-4 weeks postanthesis) on MS medium with 4 mg/l 2,4-D and 0.25 mg/l BAP for 1 week prior to transfer to MS basal medium (Merkle and Wiecko 1989, Merkle 1991). Clumps of somatic embryos emerged from the immature seeds, apparently after forming directly from tissues of the zygotic embryo. New somatic embryos continued to be produced for some time via direct, repetitive embryogenesis from the radicles of older embryos. However, repetitive embryogenesis was terminated when the embryos germinated precociously, which also resulted in spindly plantlets. Precocious germination was limited by subculturing clumps of somatic embryos to MS medium with the sucrose concentration raised from 3% to 10% and by interposing a layer of filter paper between the embryos and the semisolid

medium, thus changing the osmotic environment of the embryos. As a result of the changed culture conditions, proembryogenic masses (PEMs; Halperin 1966) were produced by the somatic embryos. PEMs may be thought of as very early stage proembryos that are rapidly proliferating via repetitive embryogenesis before they develop further. We consider this form of embryogenesis to offer the best potential for very high frequency somatic embryo production and for gene transfer applications (see section on yellow-poplar). PEMs rapidly proliferated in either semisolid or liquid 10A40N medium (Finer and Nagasawa 1988), a medium originally developed for soybean suspension cultures, supplemented with 3 mg/l 2,4-D (Merkle 1991). Hundreds of black locust somatic embryos were produced by transferring approximately 0.5 g of PEMs to hormone-free liquid 10A40N medium (Arrillaga and Merkle, unpublished). Embryos were germinated either on 1/2-strength MS medium or, at low frequency, directly on peat/vermiculite potting mix in GA7 vessels (Magenta Corp.).

Somatic embryogenesis has also been reported in other North American woody legumes, including eastern redbud (*Cercis canadensis*; Trigiano et al. 1988, Trigiano and Beaty 1989, Geneve and Kester 1990) and yellowwood (*Cladrastis lutea*; Weaver and Trigiano 1991). In all cases, the explants were immature embryo tissues. Redbud embryogenesis is of special interest since somatic embryos were formed directly from cotyledons (Trigiano et al. 1988), indirectly from callus (Trigiano and Beaty 1989) and even from adventitious roots (Trigiano, unpublished).

Oaks and Chestnuts

In vitro propagation of members of the Fagaceae has been difficult, with virtually no reports of any method with the potential for application for mass propagation. However, we believe recent progress with somatic embryogenesis with members of this family is noteworthy, especially considering the high value of products derived from these species, in particular the genus *Quercus*. In the past few years a number of European oak species have been regenerated via somatic embryogenesis, including English oak (*Quercus robur*; Chalupa 1990), Durmast oak (*Quercus petraea*; Chalupa 1990) and cork oak (*Quercus suber*; Bueno et al. 1992). Among North American oak species, Gingas and Lineberger (1989) regenerated northern red oak (*Quercus rubra*) plantlets via somatic embryogenesis. Although the highest frequency somatic embryo production was achieved from immature (late heart to early cotyledonary stage) zygotic embryos cultured on MS medium with 1 mg/l 2,4-D and 1 mg/l BAP, the highest number of normal, bipolar embryos resulted from culturing the explants on hormone-free MS medium. A few embryos were induced to convert to plantlets by desiccating them inside empty, sealed Petri plates for 2-3 weeks, apparently allowing them to break epicotyl dormancy. Embryogenic cultures of white oak (*Quercus alba*) were generated using the same procedure, although no plantlets were generated. Gingas (1991) reported production of somatic embryos from partially expanded male catkins of swamp white oak (*Quercus bicolor*) cultured on MS medium with 1 mg/l 2,4-D. Callus which proliferated from the base of individual male flowers produced somatic embryos following transfer to MS medium with 1 mg/l BAP. Normal, bipolar embryos transferred to 1/2-strength MS basal medium produced plantlets. This report is of particular interest because it is one of the few demonstrating that embryogenic cultures can be initiated from mature tree tissues (see section on advances expected in the near future).

There is renewed interest in research with the American chestnut (*Castanea dentata*), which was virtually eliminated from the eastern forests of the United States during the first half of the 20th century by chestnut blight, caused by the fungus *Cryphonectria parasitica*. Part of this effort is the establishment of in vitro regeneration systems for future application in clonal propagation of resistant genotypes and for genetic engineering purposes. Micropropagation procedures have been reported for immature tissues by Keys and Cech (1982) and for mature tissues by Read et al. (1985), who cultured 5 cm apical portions from forced buds on WPM supplemented with 0.5 mg/l BAP. Recently, Read (1992) reported improved axillary shoot production from American chestnut cultures by substituting low concentrations (0.05 μM) of TDZ for BAP. Merkle et al. (1991) initiated embryogenic American chestnut cultures from immature seeds cultured on WPM with 4 mg/l 2,4-D and 0.25 mg/l BAP. PEMs, apparently derived from the immature zygotic embryo, transferred to hormone-free WPM developed into cotyledonary embryos, but no plantlets were produced.

Yellow-Poplar

Somatic embryogenesis in yellow-poplar (*Liriodendron tulipifera*) tissue cultures was

reported by Merkle and Sommer (1986). Immature zygotic embryos cultured on a Blaydes' (Witham et al. 1971) induction medium with 2 mg/l 2,4-D and 0.25 mg/l BAP produced a fast-growing, nodular callus, since reclassified as PEMs. Similar to many embryogenic systems, PEMs maintained on induction medium continued to proliferate without further development, but transfer to hormone-free (basal) medium allowed somatic embryos to develop from the PEMs. Embryos converted to plantlets at a low frequency, following transfer to a modified Risser and White's (1964) plantlet development medium.

Since the first report, the yellow-poplar embryogenic system was improved in two ways. First, by initiating cultures from developing zygotic embryos collected at regular intervals throughout the summer, it was found that the optimum stage of development for initiation of an embryogenic culture was the late globular to early heart stage, which occurred approximately 8 weeks postpollination in the southeastern U.S. (Sotak et al. 1991). By applying this information, embryogenic cultures were routinely obtained from up to 50% of explants (Merkle, unpublished). Secondly, the flexibility inherent in cultures that proliferate as PEMs was exploited to scale-up production of somatic embryos with a high potential for conversion to plantlets (Merkle et al. 1990, 1991). Embryogenic yellow-poplar suspension cultures were initiated by transferring PEMs to liquid induction medium in Erlenmeyer flasks and maintaining them on a gyratory shaker, with transfer to fresh medium every 3 weeks. Although large numbers of somatic embryos were obtained by transferring size-fractionated PEMs to liquid basal medium, conversion of these embryos to plantlets was very low (Merkle et al. 1990). Therefore, a protocol was adopted in which PEMs were size-fractionated on stainless steel screens and the 38 μm - 140 μm fraction was collected on a disk of filter paper using a Buchner funnel. Then, the filter paper with PEMs was plated on semisolid induction medium. Within 2-3 weeks, a roughly synchronous population of mature somatic embryos developed from the PEMs on the filter paper. Embryos transferred from the filter paper to basal medium lacking casein hydrolysate formed plantlets at frequencies up to 84%, depending on the clonal line (Merkle et al. 1991).

The high-frequency plantlet production of the fractionation/plating procedure offered the opportunity to initiate the first large-scale field test of somatic embryo-derived trees of a southern hardwood. PEMs representing 9 embryogenic lines were increased for embryo production by growing suspension cultures in 250 ml Erlenmeyer flasks. Suspension cultures were size-fractionated and plated as described above and over 1000 somatic embryos of each of the clones were produced and shipped to cooperators at the University of Tennessee for germination, plantlet development, transplanting to soil and acclimatization. As embryos germinated, they were transferred to plantlet development medium in GA7 vessels and grown for 2-3 months in a growth room. Plantlets were transplanted to a peat/vermiculite potting mix in planting containers, grown in a misted greenhouse for 2-3 months and transferred to the greenhouse. By the end of the growing season, of approximately 8700 plantlets transferred ex vitro, about 5500 (63%) were hardened off and transferred to the shadehouse where buds were set in the fall. Over 99% of the plantlets broke dormancy and continued growth the following spring (Merkle et al. 1991). Plantlets were maintained for another season in the shadehouse and planted in replicated field tests using both clonal block and clonal mix designs in March-April, 1992.

Although not directly related to propagation, it should also be noted that growth of PEMs in suspension culture facilitated development of a system for efficient production of transgenic yellow-poplar trees (Wilde et al. 1992). Embryogenic yellow-poplar suspensions, size-fractionated to enrich for single cells and small cell clusters less than 140 μm in diameter, made excellent target material for gene transfer via microprojectile bombardment. Antibiotic-resistant callus colonies were maintained under strict selection in antibiotic-supplemented liquid medium to block growth of nontransformed cells, virtually guaranteeing production of nonchimeric embryos and plantlets. Thus, due to the great potential for manipulation offered by embryogenic cultures (i.e. growth as suspensions and ability to size-fractionate) nonchimeric transgenic trees were efficiently generated.

Earlier, rare hybrid trees were used as an example of a value-added feature potentially available via tissue culture propagation. Hybrids between the North American yellow-poplar (*L. tulipifera*) and the Chinese tuliptree (*Liriodendron chinense*) were reported to be heterotic, displaying

growth rates superior to those of either parent (Parks et al. 1983). Seedling-derived trees of this hybrid, however, remain rare in the U.S., due to the availability of very few Chinese tuliptree parents. To test the applicability of embryogenic cultures for propagation of rare hybrid trees, cultures were initiated from immature embryos resulting from controlled pollinations of *L. tulipifera* female parents with pollen collected from one of the few mature *L. chinense* trees in the U.S.. Media and conditions were the same as described by Merkle and Sommer (1986). Averaged across the 4 hybrid families, 12% of the explanted embryos produced embryogenic cultures. Unlike pure yellow-poplar embryogenic cultures, however, hybrid cultures tended to proliferate as clusters of repetitive globular embryos, rather than as PEMS. Thus, none of the hybrid lines was amenable to growth in suspension culture, limiting large-scale propagation. Nevertheless, approximately 100 embryos from different hybrid lines were converted to plantlets, some of which displayed rapid growth following transfer ex vitro (Merkle et al. 1992).

ADVANCES IN IN VITRO PROPAGATION EXPECTED IN THE NEAR FUTURE

Although a number of authors have speculated on the future impact of in vitro propagation on forestry, this discussion is focused on a few potentially-important advances which may impact in vitro propagation of hardwoods in the short term. First, a number of recent reports have appeared on the promotive effects of plant growth regulators that have not been widely used with hardwood species previously, notably phenylurea compounds with cytokinin-like activities, such as TDZ and CPPU [N-phenyl-N'-(2-chloro-4-pyridyl) urea]. These compounds have been shown to have strong developmental effects, especially with regard to adventitious shoot production, with a number of hardwood genera, including *Castanea* (Read et al. 1992), *Fraxinus* (Bates et al., in press), *Juglans* (Neuman et al., in press) and *Populus* (McCown et al. 1991). It is likely that additional work with application of these growth regulators for in vitro propagation of hardwoods will be reported in the near future.

Other major advances concern somatic embryogenesis, which probably has the highest probability of ultimately providing propagules on an economical scale. First, it is likely that success will be achieved with somatic embryogenesis from mature tissues (or at least tissues of known genetic value) of hardwood species in the near future. Already there are reports of somatic embryogenesis from male flower parts of *Quercus* (Gingas 1991) and *Aesculus* (Jorgensen 1989). Also, Michler and Bauer (1991) used leaf tissues of a *Populus* hybrid of known genetic value to obtain somatic embryos. Similarly, as more cultures are initiated from male flower parts, there will likely be reports of embryogenesis from haploid tissues (androgenesis), generating either haploid lines, or homozygotic diploid lines which would prove useful in tree improvement.

Another area which is likely to come into widespread application for operational propagation of hardwood trees is long-term storage of embryogenic cultures, primarily using cryopreservation. Since most of the embryogenic systems reported to date depend on material of unknown genetic value, trees derived from these cultures need to be tested for field performance before being released for production programs. Even if researchers are eventually able to clone mature genotypes of a particular hardwood species via somatic embryogenesis, there will still be a need for field evaluation of trees derived from these cultures. However, under continuous culture, the useful life of most embryogenic cultures does not exceed a few years. Therefore, embryogenic cultures must be held in a suspended state while trees derived from them are tested. Once field testing is complete, cultures of those clones with the best field performance can be scaled-up for production. Fortunately, embryogenic cultures of angiosperms seem to be very amenable to cryopreservation, and this technique has already been applied for long-term storage of embryogenic cultures of *Betula* (Mannonen and Monger 1992).

CONCLUSIONS

In vitro propagation of hardwood trees has a long history, and has progressed over the past few decades, despite the relatively small research effort in this area. With a few exceptions, most in vitro propagation systems for southern hardwoods are not adequate for routine clonal propagation of trees, due to low multiplication rates, high labor costs, limitation to genetically unproven material, or other features that make them economically uncompetitive with seedlings or macropropagation. A few systems summarized here demonstrate that in vitro propagation has the potential to be applied for

operational propagation programs. As techniques are improved to raise the frequency of plantlet production, lower labor costs, guarantee clonal fidelity, propagate mature genotypes, and integrate value-added traits into propagules, it is likely that in vitro propagation systems will be increasingly applied for operational propagation of hardwood forest trees.

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REGENERATION OF THE GENETICALLY ENGINEERED CONIFER - THE IMPORTANCE OF THE BIOLOGICAL SYSTEM[1]

David D. Ellis, Alex M. Diner and Yinghua Huang[2]

Abstract.--Genetic engineering of forest trees offers a unique opportunity to selectively add single important traits to superior trees while still maintaining the mix of genes responsible for the superior phenotype. Within the conifers, the only two examples of stable regeneration of transformed plants include transformation of *Larix decidua* by *Agrobacterium rhizogenes* and transformation of *Picea glauca* by particle acceleration. Reliance on identifying particular attributes of the biological system into which the DNA is delivered, has played an important role in the development of these two transformation systems. While the precise attributes that are important for allowing gene transfer are unknown, numerous factors such as the meristematic state of the explant, the rate of cell division and the age of the explant all play a role[3].

INTRODUCTION

Genetic engineering of forest trees offers several possibilities to complement traditional tree breeding programs. Genetic engineering technology can selectively add a single trait to superior trees while still maintaining the existing combination of genes responsible for the superior phenotype. Then, with the use of vegetative propagation methods, genetically engineered individuals can be clonally multiplied. Although there are few genes currently available for traits important to forest trees, the outlook is promising that genes for such traits as lignin biosynthesis, specific gravity, and pest resistance will be available in the near future. Unfortunately, methods to genetically transform forest trees are available for only a few species, including *Populus* (poplar) (Fillatti et al. 1987, Pythoud et al. 1987, McCown et al. 1991), *Lireodendron tulipifera* (yellow poplar) (Wilde et al. 1992), *Larix decidua* European larch (Huang et al. 1991) and *Picea glauca* (white spruce) (Ellis et al., submitted). This limited success is not due to the inability to insert foreign DNA into plant cells; rather it is due to the scarcity of tissue culture systems for the regeneration of plants containing the inserted genes.

There are only two examples within the conifers, of regeneration of genetically engineered trees. Transformed *Larix* plants were regenerated using *Agrobacterium*, a plant-pathogenic bacterium which during infection transfers a segment of its DNA into the plant genome (Huang et al. 1992). In this case, the apparently high susceptibility of seedling tissues to inoculation with *Agrobacterium* and the competence of those tissues for hairy root or shooty tumorigenesis proved critical for success. Genetic transformation of *Picea* was achieved using microscopic gold particles which were coated with DNA and propelled into cells by a process known as particle acceleration (Ellis et al.,

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submitted). In this case, the identification of a specific stage of embryo development was important not only for the regeneration of transformed plants, but also for the expression of the introduced genes. Clearly, the reliance on the identification of particular developmental stages of plants used for transformation studies is crucial not only for expression of foreign genes, but also for the ability to regenerate transformed plants.

Successful transformation of plants depends on matching several biological determinants with many physical constraints. With the various gene transfer methods currently available, simple placement or transfer of DNA into a plant cell is no longer a limiting factor. However, targeting of the DNA to a particular cell within a complex tissue or organ competent for regeneration is a major limitation. Further, microculture methods which complement gene transfer technology do not exist for most plants. Even when a microculture method does exist for a particular plant, the identity of those cells which divide to form new plants is often unknown. In addition, the cell containing the DNA must be competent to express the introduced genes. This paper discusses these areas of gene transfer in relation to coniferous forest trees, and speculates on the different causes and effects which cellular biology may have on the success of gene transfer.

GENE TRANSFER

Genes which are transferred into plants, are placed into single cells. Simple penetration of the cell wall and plasma membrane is only a small portion of the process. The DNA must then pass through the cytoplasm and the nuclear membrane, before finally arriving intact in the nucleus. In order for a stable transformation event to occur, the DNA must then be incorporated into the plant's genome. Although it is known that all of these events must happen, the mechanisms involved from the entry into the plant cell to the integration into the plant genome are unknown. However, the use of inoculation/regeneration-competent cells is critical; thus: the need to determine the temporal "window of competence" for such cells.

Once integrated into the genome of a particular cell, those genes on the inserted sequence must be expressed in order to establish a functional transformation event. Expression of the inserted genes depends on several factors including 1) the site of insertion and the copy number of the inserts, 2) the promoter or regulatory element driving the gene, 3) the gene itself , 4) the specific types of tissues or organs for inoculation, and 5) the physiological and developmental state of the transformed cell. Although each of these factors is discussed briefly, focus is placed on the fourth variable, the state of the recipient cell.

GENE INSERTION

There is some evidence that introduced genes can be targeted to a particular site in the genome by homologous recombination; yet in general, the site of DNA insertion is assumed to be random. Although insertion may be random, the effect of (1) those DNA sequences flanking the introduced DNA, (2) the number of copies and (3) the sequence of the introduced DNA will all affect gene expression. The expression level of the introduced DNA was postulated to be higher if the DNA were integrated into, or close to an area in the genome which is being actively transcribed. Although the precise mechanisms by which the flanking genomic sequences affect the expression of the introduced DNA is unknown, it is clear that certain regions of the genome, such as the condensed regions containing heterochromatin, are not actively transcribed. It therefore follows that DNA inserted into these regions will also not be actively transcribed. Further, inserted endogenous promoters might override regulatory elements used to drive the introduced genes. Although little experimental evidence exists to explain the influence of endogenous flanking DNA sequences on the expression of introduced DNA, the influence of the endogenous DNA on the expression of inserted DNA is generally referred to as the "position effect".

CO-SUPPRESSION

Multiple copies of genes were also been shown to influence introduced gene expression. Known as "co-suppression", this phenomenon accounts for the suppressed expression of introduced DNA when either multiple copies of a gene are inserted into the genome, or when introduced DNA encodes for a gene already present in the genome. The best example of this latter case is in *Petunia* where introduction of an exogenous chalcone synthase (CHS) gene shut off expression of the endogenous gene (Napoli et al. 1990). In some cases, methylation of the DNA is associated with co-suppression; yet this has not been shown to be responsible for the suppression of gene expression in all cases of co-suppression.

PROMOTERS

Each somatic cell within a plant theoretically contains the same complement of genes. What differentiates a leaf from a stem or flower is the expression of which genes are turned on or off in the cells in these tissues or organs. The regulatory sequences of DNA responsible for the expression of one set of genes and not another are known as promoters. Promoters are sequences of DNA adjacent to the gene which contain the necessary information to precisely regulate the expression of the gene so that it is expressed only in the proper cell type and at the proper time during development. It has been generally assumed that a specific regulatory sequence, or promoter, can be used to express any gene attached to it in the manner programmed by that promoter. Therefore, the expression of introduced genes could be restricted to certain tissues or developmental stages. Clearly, the promoter used to drive the expression of the introduced gene plays a major role not only in the overall level of gene expression, but also in the location within a regenerated plant in which the genes will be expressed. While there are no reports on the differential expression of genes by different promoters in transformed forest trees, numerous studies have investigated the expression of various genetic constructs using transient assays (Bekkaoui et al. 1990, Wilson et al. 1989, Ellis et al. 1991).

Since the mechanisms responsible for expressing genes are present throughout the nucleus, an introduced gene can be expressed prior to stable integration into the genome. Such expression is known as transient gene expression. Therefore, with transient gene expression assays, the expression of different promoters can be tested quickly in various cell types without the need for stably incorporating the DNA into the genome. The abovementioned studies on transient expression in conifers have confirmed the expression of a number of angiosperm promoters. The ability to use angiosperm promoters to express genes in conifers demonstrates the universal nature of the genetic code and opens the door for differential regulation of inserted genes in conifers. This is crucial because few conifer promoters have been isolated.

While these studies have shown that angiosperm promoters function in conifers, transient assays have failed to demonstrate tissue-specific expression (Ellis et al. 1991). The ability to rapidly test for tissue- or cell-specific expression using transient assays would be a very powerful tool, as the differential expression of genes in long-lived perennials may be very useful. It is important to note, however, that although these promoters do not function in a tissue-specific manner in transient assays, they may express a gene in a predicted inducible or tissue-specific manner once stably integrated into the plant genome. This has been the case in herbaceous plants where promoters did not give the predicted inducible or tissue-specific expression in transient assays, but showed proper expression when stably integrated (D. Russell, personal communication). There is no reason to expect that the predicted inducible or tissue-specific expression from these promoters would not occur in forest trees once genes are stably integrated.

To date, all the examples of stable integration of introduced DNA in forest trees have utilized promoters which are not generally expressed in an inducible or tissue-specific manner. Examples of these are cauliflower mosaic virus (CaMV) 35s promoter and nopaline synthase (nos) promoter (McCown et al. 1991, Ellis et al., submitted). As transformation systems are developed for forest trees, the testing of numerous promoters in stably transformed plants will surely follow.

STABLE GENE TRANSFER USING AGROBACTERIUM

Several studies have focused on the identification of *Agrobacterium* strains which infect conifers (Sederoff et al. 1986, Clapham et al. 1986, Ellis et al. 1989, Morris et al. 1989, Stomp et al. 1990, Huang et al. 1991, Huang and Tauer, personal communication). These studies demonstrated some important factors for the application of *Agrobacterium* for gene transfer in conifers including, 1) a high degree of variation in infectivity by different *Agrobacterium* strains; 2) infectivity is different for different conifer species or genotypes; 3) infectivity is dependent on the developmental stage of the tissue inoculated. The original approach to *Agrobacterium* work in conifers was to confirm that *Agrobacterium* would infect conifers similar to other plants and then to identify *Agrobacterium* strains which could infect conifers at a high frequency. As a serendipitous by-product of this approach, developmental stage-specific interactions affecting *Agrobacterium* infection were identified. For example, all *Agrobacterium tumefaciens* strains infectious to white spruce seedlings infected young tissue at a higher frequency than older tissue (Ellis et al. 1989). A number of these strains also had a higher frequency of infection on the younger tissue of both *Picea engelmannii* (Engelman spruce) and *Picea sitchensis* (Sitka spruce).

Infection by *Agrobacterium* of younger actively growing tissues is not unique to spruce and was reported in other conifers (Diner and Karnosky 1987, Morris et al. 1989, Stomp et al. 1990). Possible explanations for the higher incidence of infection in the younger tissue could be a lack of resins in younger tissue (Diner and Karnosky 1987), differential phenolic production following wounding in old vs. young tissue (Ellis et al. 1989), the relative level of stem woodiness (Stomp et al. 1988) and differences in the rates of cell division (A. Stomp, personal communication). Interestingly, the level of *Agrobacterium* infection was the same in both young and old tissue in *Pseudotsuga menziesii* (Douglas-fir) (Ellis et al. 1989), suggesting that older tissue in Douglas-fir does not undergo the same rapid changes associated with maturation that occur in the other species. In addition to species and ontogeny specificity, successful inoculation with agrobacteria is also genotype-dependent. Recent results from studies of loblolly pine genotype transformation potential suggest that host genotype moderates infection (Huang and Tauer, personal communication).

LARIX AS A CONIFER MODEL FOR *AGROBACTERIUM* INFECTION

Larix decidua has proven to be very amenable to transformation, tumorigenesis and the regeneration of transformants using *Agrobacterium* vectors. Preliminary attempts in 1985 to inoculate *L. decidua* germlings with *A. rhizogenes* pRi11325 resulted in the differentiation of tumorigenesis within 30 days. The choice of germlings was deliberate (Diner and Karnosky 1987), although use of older hosts was later attempted (Huang et al. 1991). The extreme susceptibility of succulent, rapidly growing tissues of other conifers to inoculation with agrobacteria has since been more broadly investigated (Ellis et al. 1989, Huang and Tauer personal communication, Morris et al. 1989). In *Larix*, tumor morphology at inoculated apical wounds included multiple shooty clusters, later shown (Huang et al. 1991) to be transformed regenerants. Tumor morphology was specific to the site of wound-inoculation. Inoculation of a very shallow wound through the center of the shoot apex induced a large, confluent teratoma-like mass of tiny buds, all of which failed to develop further (A. Diner, unpublished). Inoculation of the apex wounded more deeply and through the cotyledonary axis resulted in up to 10 progressively developing shoot buds, while only roots grew from inoculated wounds made to the lower hypocotyl. These specific tumor types could form either individually on an appropriately inoculated germling, or collectively on a single germling inoculated with pRi 11325 at the three respective sites.

In contrast, wound-inoculation of *L. decidua* germlings with *A. rhizogenes* A4pARC8 (Simpson et al. 1986) at any of these same three sites elicited only root tumorigenesis (A. Diner, personal communication). Frequency of tumorigenesis was virtually 100%, similar to that using pRi11325; transformation was verified by Southern blot analysis (Y. Huang, personal communication). Only infrequent host death by unidentified causes, precluded confirmation of successful transformation.

Transformed regenerant plants are not uncommon consequences of tumorigenesis induced either by *A. tumefaciens* or *A. rhizogenes* (Ondrej et al. 1984, Ondrej and Biskova 1986, Shahin et al. 1986, Jouanin et al. 1987, Noda et al. 1987, Morris and Robbins 1992), while spontaneous, well-defined shoot organogenesis from in situ *Agrobacterium*-induced tumors is more rare.

Shoot or root tumorigenesis may be a result of concentrations and proportions of phytohormones to which the transformed cells are exposed in their microenvironment, as well as to the duration of that exposure. Differential morphogenesis from cell cultures or organized tissue explants has been classically described to be under phytohormone control. It is unlikely that the organogenic role of a phytohormone is direct; control of the organ-initiating event is more likely mediated by substances whose syntheses are promoted or inhibited by the respective phytohormone(s). Indeed, both those transformed tissues (roots and shoots) which are phytohormone-independent and those normal tissues to which phytohormones are exogenously applied elaborate novel transcription products, several of which appear to be shared (Burrell et al. 1986). In addition to the phytohormone independence conferred by transformation (Klee et al. 1987), there is an enhancement of tissue sensitivity to phytohormones (Maurel et al. 1991). Organogenesis may thus be an almost spontaneous consequence in a population of cells transformed both to auxin independence and extreme sensitivity.

Studies suggest that tumorigenesis and tumor morphology are controlled by 4 factors: (1) the plasmid (Klee et al. 1987), (2) the host (Delmotte et al. 1991), (3) their interrelationships (Stachel et al. 1985), and (4) the site of *Agrobacterium* inoculation of the host plant (Gresshoff et al. 1979, Huang et al. 1991) which may be merely localized anatomical expressions of factor three. Certainly, there are physical and temporal gradations

in the availability of all pertinent phytohormones in the shoot. The concentration gradients of auxin and other phytohormones in the shoot may thus be responsible for effects on morphology (Burrell et al. 1986) and gene transcription (van Slogteren et al. 1984) in transformed shoots grafted to normal stems, and may help to explain the site-specific *Larix* tumor morphology developed from inoculation with *A. rhizogenes* pRi11325, as described above. Beyond cell competence for differentiation and regeneration, tumor morphology in some hosts depends upon the specific plasmid employed (Huang 1991). In the case of the two most studied Ti plasmids, the octopine type induces undifferentiated tumors , while the nopaline type induces teratomous tumors with some shoot differentiation. Generally speaking, transformed plant cells carrying wild-type T-DNA of an armed Ti plasmid are unlikely to be regenerated to plants. However, successful examples of plant transformation and regeneration using oncogenic strains of agrobacteria have been reported in a variety of angiosperm species (Tepfer 1990, Yang and Simpson 1981). Further use of the *A. rhizogenes* Ri plasmids is relatively straightforward for regeneration of whole plants following tissue transformation, as compared to the Ti plasmid. As we see it, *A. rhizogenes* strain 11325 is a special strain, because it carries the nopaline-type Ri plasmid. The pRi11325 is a unique system for gene transfer and recovery of transformants via spontaneous shoot organogenesis in European larch (Huang et al. 1991).

That very young (nonwoody) conifer tissues appear so susceptible to inoculation and transformation by *Agrobacteria* (Diner and Karnosky 1987, Morris et al. 1989), may be less a consequence of the absence of bactericidal secondary products in those tissues, than the simple availability of viable, physiologically dynamic tissues to which a particular strain of *Agrobacterium* may be virulent. Cell cycle phases exert considerable effects on transformation efficiency using particle bombardment (Iida et al. 1991). Synchronous cells bombarded at M and G2 phases were transformed at 4 to 6 times the frequency of those bombarded at S and Gl phases. In order to develop effective protocols for integrative transformation and regeneration, some studies have focused on the use of meristematic cells as targets (Potrykus 1991). These types of cells have been shown competent for transformation, but are often not competent for regeneration in many plant species generally considered recalcitrant to that process. This may suggest that competence for transformation is not pertinent to competence for regeneration. Possibly, a specific cellular developmental stage is liable to differentiation, particularly in response to some trigger. In the case of spontaneous organogenesis following transformation, we speculate that a "window of competence" exists for a cell, tissue or organ, and is a specific and short fraction of a developmental stage. Thus, synchronous cells may be collectively competent for gene transfer and regeneration (Huang 1991). This may be one plausible explanation for initiation of adventitious shoot buds from transformed cells of European larch.

Certainly, the mere fact that cells may be in synchrony ought not to be pivotal to their transformation frequency in *Larix* apices or hypocotyls inoculated with *Agrobacterium*, though the juvenility and active growth of germlings implies that some synchrony likely occurs at least adjacent to the meristem, potentiating the availability of cell aggregates collectively undergoing common phases of division. The transformation and early cell "commitment" to specific tumor morphology in any of these aggregates may regulate similar commitment by other transformed cells in that or another aggregate. There are suggestions that pre-existing primordia influence the character of primordia formed later (Poethig 1991).

However, another form of "synchrony" appears to be maintained in older tissues of woody species, causing rhythmic and direct effects on cambium division and differentiation (Stieber 1985). Stieber suggested that unidentified, mobile "function-determining factors" act in synergy with auxin to control cell differentiation. These factors, if real, may also affect tissues of the germling such that their concentration(s) at any locus synergistically controls the early differential morphologic development of organs from a transformed cell.

STABLE GENE TRANSFER USING PARTICLE ACCELERATION

To achieve transformation using particle acceleration, microscopic gold or tungsten particles are coated with DNA and shot into cells. Although generally inefficient in yielding stable integrative transformation, one major advantage of this technique over other direct gene transfer techniques is that particle acceleration allows the transfer of genes into cells intact in tissues. Theoretically, DNA can be inserted into any cell which is impacted by the particle. Foreign genes were expressed in all conifer tissues exposed to particle acceleration including embryos, seedlings, megagametophytes, xylem, pollen, needles, buds, cell suspension cultures, embryogenic callus, cell

aggregate cultures and roots. While almost all of this expression was transient, it yielded valuable information on factors involved in the expression of introduced genes in the various tissues competent for regeneration. For example, freshly excised embryos of white spruce expressed introduced DNA at a relatively low level. Pretreating the embryos on bud induction medium containing cytokinin (Ellis et al. 1991) prior to particle acceleration resulted in a dramatic increase in the level of gene expression. This increase continued for up to seven days of pretreatment, and then stabilized so that a longer pretreatment period was ineffective. Clearly, whatever change was induced by the bud induction treatment which was responsible for the increase in gene expression, occurred within seven days.

The cellular changes which occurred on bud induction medium were well documented for numerous conifer species. Within this seven day period, changes in cell division patterns from anticlinal to periclinal were observed which resulted in the proliferation of meristemoids. These meristemoids continued to divide, and within seven days the epidermal and subepidermal cell layers associated with the meristemoids contained localized areas of densely cytoplasmic isodiametric cells characteristic of meristematic cells. The induction of meristematic tissue was suggested to be important for the expression of introduced genes in spruce (Ellis et al. 1992), poplar (McCown et al. 1991) and *Vaccinium* (cranberry) (Serres et al. 1992). The dependence of gene expression on a specific physiological and developmental stage of the cell may relate to the above discussion concerning *Agrobacterium*, in which both biochemical changes and cell attributes such as cell division rates were thought to be related to the ability of *Agrobacterium* to infect tissues.

Whether the expression of foreign genes in woody plants is directly related to meristematic cells and whether the cells which are induced with a pretreatment are truly "meristematic" have been debated. The important issue, however, involves identification of those cellular attributes which account for their higher competence to express introduced genes. If the analogy to a meristematic state is continued, it can be assumed that these cells have the ability to rapidly divide, and that they have a high rate of metabolic activity. These two factors may be related. Further, a cell with high metabolic activity would likely be active in endogenous gene expression with all the functions for gene transcription and translation actively expressed. It would only follow then, that genes introduced into such a cell would also have a higher probability of being expressed.

How this may be related to the regeneration of stably transformed plants is that 1) DNA replication may aid in the incorporation of foreign DNA, and 2) expression of introduced genes is required for selection of transformants based on antibiotic selection. There is indirect evidence that a certain phase (S phase) in the cell cycle may be a prerequisite for DNA integration into the genome. Possibly, the importance of this DNA synthesis phase involves many DNA repair and synthesis mechanisms which are active during this phase and which may aid in the incorporation of the DNA into the genome. Unfortunately, these experiments are difficult both to perform and interpret. Therefore this topic remains a black box. In contrast, the expression of introduced genes is definitely required for selection. The incorporation of a gene encoding for resistance to an agent (antibiotic or herbicide) which is normally lethal to a plant cell is critical to the identification of a single cell containing the introduced DNA in a large population of untransformed cells. If the gene encoding resistance to the selective agent were not expressed, selection for that cell containing the inserted DNA would not be possible.

PICEA AS A CONIFER MODEL FOR PARTICLE ACCELERATION

The time course of gene expression over an eight week period following DNA insertion by particle acceleration was investigated in white spruce (Ellis et al. 1992). The time course of expression with numerous promoters is characterized by a high initial level of gene expression which is maintained for seven days. Fourteen days after particle acceleration, however, this expression decreased dramatically to a lower level of expression which was maintained for up to eight weeks (56 days). This type of decay in gene expression may allow for the selection of transformed cells in a rapidly dividing plant system such as in *Nicotiana tabacum* (tobacco). But in conifers which grow considerably slower in culture, it is desirable to maintain maximal gene expression over a longer period of time. Interestingly, this characteristic time course of gene expression remained unaltered with many different cultural treatments. Some treatments, such as placing the tissue at cooler temperatures (22°) after particle bombardment altered the baseline level of gene expression, yet had no affect on the decline of gene expression after 7 days.

This rapid decline in gene expression was first thought to represent the degradation of DNA in the cell prior to its incorporation into the genome. However, using an inducible heat shock promoter, it was discovered that maximum gene expression was induced for up to four weeks, suggesting that the DNA was remained intact and that expression of the introduced DNA was somehow shut off. This theory was recently supported by the use of a heat shock, which resulted in maximal expression activity of other promoters maintained beyond the initial 7 day period. The phenomena which caused the decrease in gene expression following particle acceleration are presently unknown. Yet, based on enzyme stability, it is known that the introduced gene was probably transcribed during the first few days following introduction into the plant cell. Further, 5-azacytidine has no effect on the time course of expression, suggesting that methylation (or azacytidine reversible methylation) is not the cause of the loss of gene expression.

As discussed above, the developmental and/or physiological stage of the target explant plays a major role in gene expression. It is important to keep in mind that the recovery of stable transformants is "a numbers game", since only a small percentage of the cells containing the introduced DNA will express the genes. And only a small percentage of those cells expressing the introduced DNA will stably integrate the DNA into the genome. Therefore, anything that is done to enhance the probability of selecting out the cells containing the integrated DNA will be advantageous. One clear advantage is to sustain maximum expression of introduced genes over a longer period. Interestingly, in contrast to zygotic embryos, the time course of gene expression in somatic embryos followed a different pattern. With somatic embryos maximum gene expression was maintained for over 3 weeks. This is further support for the reliance on a biological entity for sustained gene expression.

The difference in gene expression over time between zygotic and somatic embryos may be explained by reference to the discussion on the importance of a "meristematic" state for transformation success. Somatic embryos are more physiologically juvenile than mature zygotic embryos. Since the former have not undergone dehydration, many of the changes associated with embryo maturation are incomplete. Although synthesis of storage proteins is initiated in later stage somatic embryos, these embryos can be regarded as immature, as evidenced by their ability to form embryogenic callus at a high frequency. In early work on the initiation of embryogenic callus from white spruce, it was found that immature embryos were the most competent to form embryogenic callus (Becwar et al. 1987). This supports the inference that somatic embryos are more juvenile or closer to the meristematic state, and may help to explain the maintenance of maximum gene expression in somatic embryos.

The ability to survive selection was as important as the expression of the introduced genes in the development of a transformation system for white spruce. Since embryogenic callus is not initiated until 3-6 weeks after placement on induction medium, keeping the embryo alive, while at the same time suppressing non-transformed cells from dividing was the strategy. Senescent conifer cells produce several secondary products such as phenolics, which are detrimental to the survival of surrounding cells. Therefore, if selection pressure kills non-transformed cells, the chances of recovering the few transformants are greatly reduced due to autotoxicity. Again, the developmental stage of the explant plays a major role in developing a selection method. The young precotyledonary stages of somatic embryos did not survive for four weeks when exposed to a relatively low level of kanamycin selection (5 μg/ml). In contrast, this level of selection was inhibitory, yet not lethal to later post-cotyledonary developmental stages.

The identification of the factors important for the expression of foreign genes and selection of transformants using the appropriate developmental stage of somatic embryos competent to form embryogenic callus, has resulted in development of a transformation system for white spruce (Ellis, unpublished). The system utilizes particle acceleration, and during the course of the past year more than 15 independent transformed embryogenic cell lines were differentiated. All cell lines tested expressed the ß-glucuronidase (GUS) gene and were resistant to ordinarily toxic levels of kanamycin, indicating expression of the neomycin phosphtransferase (NPT) gene. Transformed embryos and seedlings were differentiated from most of these embryogenic lines, and seedlings from two lines were transferred to the greenhouse.

A third gene which has been inserted into all of the transformed spruce lines encodes for the proteinaceous insecticidal cryIA endotoxin from *Bacillus thuringiensis* (B.t.). Preliminary evidence from feeding trials with *Choristoneura fumiferana* (spruce budworm) indicates that this gene was expressed at a low level. Parallel studies by our group in entomology have observed the feeding behavior of insects and toxicity of this protein to spruce budworm, and showed that sublethal levels of

the toxin inhibit feeding, causing a decrease in larva weight. This property of B.t. is a good indicator of B.t. expression in transformed tissues (Ramachandran et al., in press). When spruce budworms were fed either transformed embryogenic callus or seedlings, there was a significant decrease in larval weights after 6 days, compared to weights of those larvae placed on control, non-transformed tissue.

CONCLUSIONS

Development of a gene transfer technology for forest trees, particularly conifers, depends on a complex set of biological interactions which operate on the frontiers of modern molecular biology. The precise mechanisms for gene insertion to the genome of plant cells is in need of considerable study. Once inserted, gene expression depends on the interaction of numerous factors. In attempting to match and overlap these factors to optimize a transformation method, it is easy to lose sight of the importance of the biological (plant) aspect of this process. As more work is done with "recalcitrant" or underexploited species, it is clear that the developmental and physiological stage of the tissue plays a crucial role in transformation success.

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Rooted Cuttings

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MACROPROPAGATION OF CONIFERS BY STEM CUTTINGS[1]

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Abstract. Trees, particularly conifers, have not been readily propagated by stem cuttings. This document groups significant biological barriers to rooting into major problem areas, and provides examples and possible solutions from the conifer literature within each area. Barriers discussed are genotypic variation, maturation, variability within trees, inadequate testing of complex cultural influences, and poor early form. Although advances have been adequate to allow large-scale rooting in some species, macropropagation is likely to become more widely adopted when components of these biological barriers, and especially their interactions, are investigated more deeply. Four key research issues, of both biological and logistical natures, must also be resolved before cuttings of any specific species can be commercially rooted on a large scale. A commercial example of overcoming biological barriers to rooting of loblolly pine cuttings is presented.

INTRODUCTION

Growers of coniferous trees have tried to apply asexual propagation techniques to their crops for many years. Successes have been difficult, but have been achieved by identifying key physiological barriers and then adapting techniques to capitalize on or enhance natural variability. This document concentrates on attempts to propagate conifers by stem cuttings, and demonstrates how major problems have been attacked.

Specifically, answers will be provided to this question: "Why has mass propagation of coniferous trees via stem cuttings been so difficult?"

Five major barriers or problem areas are discussed: (1) the weak or variable genetic rooting capacity of many desirable species, (2) reduced rooting and growth due to maturation, (3) rooting variability among cuttings collected within a tree, (4) insufficient testing and incomplete success of cultural enhancements, and (5) poor form of rooted cuttings from some species. Since many physiological processes are interrelated, physiological influences on rooting are likewise interrelated, and these five areas are therefore not completely discrete. The authors have selected this format primarily for ease of communication and will identify interactions between topics as appropriate.

For each area, literature focusing on stem cutting propagation has been chosen to illustrate specific problems and solutions. Rather than reviewing all pertinent works within a problem area, representative data that make good examples will be discussed. Examples are world-wide, but many are from North America. These works appear within forestry, Christmas tree, and horticulture literature. A few examples use plant parts other than stem cuttings and these have been distinguished by underlining (e.g. *hypocotyls*) for clarity.

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INADEQUATE GENETIC ROOTING CAPACITIES

Problem

Many desirable species can be rooted only on an experimental scale. In reviewing research publications that address rooting, there apparently has been no definitive comparison of rooting variability among the more important conifers. However, enough information exists to categorize the various species as easy, moderate, or difficult-to-root regardless of the phenotypic traits, climatic conditions, or seasonal changes associated with vegetative propagation. This is not to say that any one species will never achieve acceptable levels of rooting, but that some species will require more intensive propagation methods. The following describes one species chosen from each of the categories to better illustrate the genetic variability in rooting.

Vegetative propagation of Norway spruce (*Picea abies* L. [Karst.]) is well established in Europe. Stem cuttings of this species form adventitious roots at their bases quite easily, and results have been well documented (Kleinschmit 1977, Bentzer 1981). Rooting rates of 95% or greater have been commonplace. Propagation methods that were described by these authors, although similar in nature, contain sufficient differences to be labeled unique, yet all have produced impressive rooting percentages.

Slash pine (*Pinus elliottii* Engelm.), a moderate species, has rooted at satisfactory percentages when exposed to outdoor nursery beds (Frampton and Hodges 1989) or controlled greenhouse environments (Reines and Bamping 1960). Rooting from hedged trees has ranged from 0% to 73%, depending on specific techniques employed (Bower and van Buijtenen 1977, Cunningham 1981). It has typically rooted within the 65-75% range for some refined trials (unpublished data, T. D. Caldwell) and is considered to be easy to root by some researchers[3]. There is no full-scale production system for this species at this time.

Loblolly pine (*Pinus taeda* L.), considered to be the most economically important of the southern pines, has the added distinction of being one of the most difficult conifers to propagate by rooted cuttings (Gardner 1929, Greenwood and Nussbaum 1981, Foster and Shaw 1987). Most attempts to root loblolly pine have generally reported successes within a range of 0%-60%. In direct comparisons with slash pine, cuttings have consistently rooted more poorly, with infrequent exceptions (Reines and Bamping 1960, Reines and Bamping 1962, Frampton and Hodges 1989). As with slash pine, the propagation methods are quite varied (Foster 1988, Foster and Shaw 1987, Greenwood and Nussbaum 1981, Frampton and Hodges 1989).

Solutions

Selection.--Despite poor overall rooting with some species, there is encouraging information showing rooting variability among full-sib families within all species tested, and even greater variability between members of the same family (Foster 1990, Anderson et al. 1992, Haines et al. 1992). Recent work (Haines et al. 1992) attempted to determine relationships between rooting and morphological features of shoots from hedges of five full-sib families of hybrid tropical pines (*P. caribaea* Morelet var. *hondurensis* Barr. and Golf. X *P. tecunumanii* (Schw.) Eguiluz and Perry). Rather than finding strong morphological markers, the genotype identity (clone) proved to be the best indicator for both greenhouse and field responses, followed by the family identity. Significant associations with morphological features will be covered later.

This information indicates that screening for families and individuals that possess strong rooting capacities can have a significant positive impact on the overall rooting success of a propagation program. Obviously, selecting for good rooting could reduce potential growth gains, but the exact impact is not known. There is evidence with western hemlock (*Tsuga heterophylla* (Raf.) Sarg.) that there may be a positive genetic correlation between rooting ability and initial growth of the rooted cutting (Foster et al. 1985). Regardless, this type of screening process can be conducted readily.

Understanding the Molecular Basis.--The long-term need is to better understand the role of gene expression in adventitious root initiation. It is fairly well established that rooting ability has a strong genetic component. Bower and van Buijtenen (1977)

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demonstrated variation among clones of slash pine. In screening several hundred loblolly clones, Foster (1990) found that a wide range of cuttings rooted. Determining what is different between a hard-to-root genotype and an easily-rooted genotype can possibly lead to the identification of the gene(s) involved in the actual rooting process. Haissig et al. (1992) have recently reviewed strategies for understanding the genetic control of rooting that would use recent developments in molecular genetics to compare rootable and non-rootable plants at the levels of transcription and translation. Initial studies most likely would not employ conifers because the described technologies are more readily applicable to the herbaceous species and woody angiosperms used to develop them. However, the implications of discoveries made in these studies should be far-reaching, and conifers should be adaptable to some of the described approaches.

Summary

The capacity to form adventitious roots under specific conditions varies widely both within and between conifer species. For desirable species that root poorly, selecting outlying families or individuals that root well can greatly improve overall propagation success, but the impact on other desirable traits is unknown. When selection can be coupled with cultural procedures that maintain juvenility and that otherwise assure strong rooting, even highly recalcitrant species should be adaptable to cutting propagation.

PRONOUNCED MATURATION EFFECTS

Problem

Poor Rooting.--Gardner (1929) was one of the first to conclude that the capacity for stem cuttings to form roots declined rapidly with tree age. Among the 21 species he attempted to root were seven conifers, including four pines. Even at that time pines were "ordinarily considered very difficult to propagate by cuttings." One-year-old wood from these pines rooted reasonably well at age one (46-98%), and much poorer two years later (0-12%). Loblolly pine was the most recalcitrant. Although data were not presented, the author also noticed that cuttings from one-year-old trees rooted faster than those from older plants.

Although Gardner established the principle of declining rooting with age, he used only limited age ranges, and a rooting environment that was only partially defined and presumably poor. Steele and others (1990) rooted dormant cuttings of Sitka spruce (*Picea sitchensis* (Bong.) Carr.) taken from the uppermost whorls of trees of various ages between 1 and 39 years. They were rooted under fairly uniform greenhouse conditions, and percent rooting data were fitted to the Gompertz equation. For this species, rooting declined dramatically between ages 4 and 11 indicating a pronounced maturation effect for this character.

Rooting is more precisely connected with phase of the cutting wood than age of the donor plant. The capacity to form adventitious roots is one of several characteristics that have been associated with the juvenile phase. As a seedling develops can occur on different parts of the same tree. In general, the lower- and inner-most areas contain tissues that remain juvenile. Therefore, rooting capacity should be expected to vary with the cutting's original position in a tree.

Grace (1939) was one of the first to demonstrate this phenomenon with conifer cuttings. Dormant cuttings were collected from the upper and lower thirds of an 18-year-old Norway spruce tree and rooted in a greenhouse propagation frame. Not only did cuttings from the more juvenile lower crown root more often than those from the upper crown (75% v. 43%), but they also produced longer roots (15 v. 9 cm). Some control over the decline in rooting with maturation can therefore be exerted by selecting cutting wood from the lower sections of trees.

Poor Growth.--Unfortunately, cuttings that have been rooted from somewhat mature material may grow slowly or be otherwise affected. Early results from a slash pine trial revealed a dramatic decline in height growth as the source for cuttings advanced in age (Franklin 1969). More recently, cuttings from seven-year-old radiata pine (*Pinus radiata* D. Don) trees were planted with 1.5/0 nursery seedlings and assessed annually for 15 years. Heights were statistically similar for both propagule types throughout the study, but diameter growth was greater for the seedlings (Klomp and Hong 1985). Further analyses (Menzies and Klomp 1988) indicated that cutting-derived trees had about 13% less volume under bark, primarily due to reduced diameter growth (Table 1). In summarizing many radiata pine studies, these authors state that there is virtually no growth reduction for cutting donors of radiata pine up to age three, but that older

sources induce slower diameter growth and poorer rooting.

Solutions

Rather than just accepting the limitation of maturation, tree propagators have attempted to improve rooting success by managing the juvenile phase of the donor material. This can be achieved in many ways, but these approaches generally involve either rejuvenation of mature material or maintenance of juvenility.

Rejuvenation.--Hackett (1985) has summarized various approaches used to attempt rejuvenation in woody plants. The most germane to mass propagation of conifers by cuttings involves, ironically, the "rejuvenation" of plants by the rooting process itself. As an example, cuttings were rooted from 11 Douglas-fir (*Pseudotsuga menziesii* (Mirb.) Franco) trees that were 12-22 years old from seed, and clonal field plantings were established from them. Three years after rooting, cuttings were collected from these trees and another set was collected from the lower crowns of the original seedling donors (Black 1972). Rooting was much greater from cuttings that originated from the grown rooted cuttings (38% vs. 11% for those from original donors) even though both donor types were genetically identical and both were the same age from seed. In addition, needle and bud morphologies were reported to have reverted to more juvenile appearances than those on the original donors. Although this approach and others may partially rejuvenate mature material, there are no consistent methods for complete rejuvenation.

Table 1. Growth of radiata pine rooted cuttings from 7-year-old trees and of 1.5/0 nursery seedlings after 15 years. Condensed from Menzies and Klomp (1988).

Response	Cuttings	Seedlings	Difference v. seedlings[1]	Statistical significance of difference[2]
Height (*m*)	19.4	19.8	-2%	ns
Diameter at breast height (cm)	34.5	37.2	-7%	**
Volume (m^3)	0.71	0.82	-13%	**

[1][(Cuttings - seedlings) / cuttings] X 100.
[2]ns=not significant, **=t-test significant at 0.01% level.

Maintenance of Juvenility.--Until rejuvenation techniques are perfected, selection and development of clones must rely on the maintenance of juvenility. One strategy is serial propagation, which theoretically uses the rejuvenating influence of successive rooting of cuttings from previously rooted cuttings to maintain high rooting potentials. Kleinschmit's (1974) early work describes extensive development of Norway spruce clones where rooting averaged 90%. Although repropagation from rooted cuttings for several "phases" did not improve rooting, it was maintained at these high levels.

Unfortunately, recent work (Dekker-Robertson and Kleinschmit 1991) indicates that subtle but important maturation does occur with serial propagation. Norway spruce clones were derived from four-year-old trees and then repropagated from rooted cuttings at three-year intervals for a total of seven serial propagation steps ("phases"). New clones were also started from new four-year-old seedlings at each three-year interval and similarly repropagated. Measurements of nursery plants, made three years after rooting, were averaged for each phase and compared with those of non-selected seedlings. Several indicators were significantly decreased by later propagation phases. For example, height at age three was reduced from about 36 cm after one propagation phase to about 32 cm after six phases. Since several measures were highest after the second propagation phase, it was postulated that the rooting process may have reinvigorated the shoot meristems, rather than rejuvenating them, presumably by bringing them closer to the root systems. The authors also demonstrated that the maturation effects could be reduced by eliminating the slowest-growing (and perhaps most mature) clones from the last three phases. The net disadvantage, however, was that it would take 10-15 years to identify superior clones and that these clones would be adequately juvenile for mass production for only another 10 years.

Shearing growth to prevent cuttings from arising from mature wood is another technique to maintain juvenile rooting. Copes (1983) began with 10- and 13-year-old Douglas-fir trees of 14 clones, pruned the lower 2 m annually, and rooted dormant cuttings from the sheared regions over eight years. Rooting capacity over all clones was not just maintained but actually increased about 3% per year to a maximum

of 67%. Therefore, meristems were again apparently partially rejuvenated or reinvigorated.

Heavy shearing of young plants to arrest maturation in cutting wood is currently practiced with several species. Experiences with radiata pine in the southern hemisphere provide a good example. Libby et al. (1972) were among the first to test earlier observations that cuttings from hedged plants rooted more like tree-form plants of the same **height** rather than of the same **age**. Plants from 68 clones were either sheared to 1-m-tall box-shaped hedges or grown in modified tree form beginning at age two. Cuttings were collected from the upper surfaces of hedges or upper crowns of trees and rooted annually for five years. By the third year, cuttings from hedges rooted significantly more often than cuttings from tree-form plants (31% v. 8%), and this difference was essentially stable for the next two years. In the fifth year the cuttings from hedges also produced significantly more roots per rooted cutting (7.4 v. 5.9).

Cuttings of both origins for 32 of these clones were planted in pairs for further evaluations (Bolstad and Libby 1982). After seven growing seasons total heights were similar for cuttings of the two origins, but bole volume was 31% greater for cuttings derived from hedges. In addition, trees of hedge origin showed more juvenile characteristics like larger branches, more lower branches, and fewer male and female flowers. Stem form was improved for cuttings from tree-form origin. Since radiata pine is grown intensively for sawtimber, the authors postulated that partial maturation could be exploited to improve timber quality. Although volume would have to be sacrificed somewhat, trunks would be straighter, and there would be fewer and smaller basal branches to remove.

Summary

Rooting of most shoots decreases dramatically with tree age. Propagators have adapted to this limitation by selecting material from the more juvenile sections of the tree, and by enhancing or maintaining juvenile rooting through propagation or shearing. Although these approaches have been somewhat successful for sustaining good rooting capacities, subsequent growth often indicates that subtle maturation has occurred.

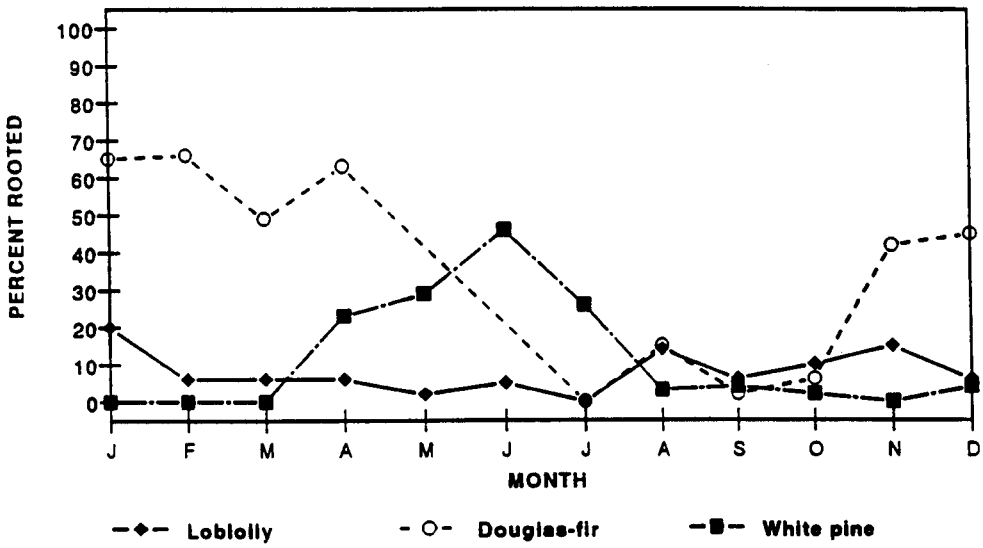

Figure 1.--Monthly rooting of cuttings from 2- to 3-year-old non-sheared loblolly pine, 8- to 10-year-old sheared Douglas-fir, and 17-year-old non-sheared eastern white pine averaged over two years. Adapted from Reines and Bamping (1960, 1962), Roberts and Fuchigami (1973), and Kiang et al. (1974).

VARIABLE ROOTING WITHIN A TREE

Problem

Given a specific set of potential donor trees of a known age, the rooting capacities of severed cuttings will change with position and time.

Cutting Position Effects.--As mentioned previously, cuttings collected from the lower crowns of Norway spruce (Grace 1939) rooted better than

those from the upper crown. Although this may be related to greater maturation in the upper crown, it is also an example of a gross positional effect. More localized effects have been found in the strongly pyramidal-shaped conifers like spruces and firs. These species have a very regular pattern of branching from the primary axis of the trunk. Data from Black (1972) generally indicated that tertiary axes from nine-year-old Douglas-fir trees rooted better (64-69%) than secondary (47%) and quaternary (41%) axes unless the tertiary axes were small (41%).

Time Effects.--Rooting success with cuttings from the same trees can also change with time. It has been generally stated that cuttings from narrow-leaved conifers in temperate regions tend to root best when collected from late fall to late winter (Hartmann and Kester 1983). For example, cuttings from eight- to ten-year-old sheared Douglas-fir rooted best between November and April (Fig. 1), depending on supplemental treatments (Roberts and Fuchigami 1973). In this test, rooting of dormant shoots was poorest in September and October when buds were most deeply dormant. It was postulated that exposing dormant shoots to cold brings endogenous biochemical promoters and inhibitors into balance for successful rooting. Cuttings from young loblolly pines also rooted best when collected in January, but rooting was generally low for this species (Fig. 1) (Reines and Bamping 1960, 1962).

There have been solid exceptions to the pattern of good cold-season rooting. Kiang and others (1974) rooted eastern white pine (*Pinus strobus* L.) cuttings from 17-year-old trees at monthly intervals for two years and found best rooting occurred with June cuttings (Fig. 1). This was associated with the presence of rapidly expanding shoots at the distal ends of one-year-old wood at the time of severance. The situation can become further complicated when secondary and tertiary axes are examined separately. Girouard (1975) tested rooting of Norway spruce cuttings collected from these two origins at two- or three-week intervals for two years. Rooting was generally most successful in mid-spring (about 53-57% for one year). Secondary axes rooted best in April while maximum rooting for tertiary cuttings occurred in May. Smaller rooting peaks for both origins occurred between September and November.

Although hedging eliminates mature upper crowns that would produce cuttings with reduced rooting capacity, the resulting juvenile shoots may still be subject to seasonal fluctuations. For example, cuttings from six slash pine clones that were four years old from seed were severed monthly from 1.2 m outdoor hedges (Bower and van Buijtenen 1977). Rooting was generally greatest for May cuttings (67%) and poorest (0%) for those collected in September. These results directly conflict with those using slightly younger, non-hedged trees where May was an intermediate month (Reines and Bamping 1960) or the poorest month (Reines and Bamping 1962), and September was an intermediate month (Reines and Bamping 1960, 1962). Although physiological changes due to hedging may be partly responsible for the discrepancy, other cultural and genetic differences are likely to have contributed greatly.

There can also be great variation in rooting from the same trees between years. When Norway spruce shoots were collected over two years (Girouard 1975), rooting was generally better in the second year of the study despite the additional age of the donor plants. This was demonstrated by the rooting maxima for lateral shoots being approximately 10-25% greater in the second year than in the first. In a study with Scots pine (*Pinus sylvestris* L.) (Boeijink and Broekhuizen 1974), the year-to-year component of variation was reported to be greater than the genetic component for rooting.

"C" Effects.--A special case of rooting variability can occur when rooted cuttings from a tree have been established as plants to produce more cuttings. This practice is critical to the development of true clones for widespread planting. Although genetically identical, rooting of cuttings can vary depending on the environmental preconditioning of individual donor plants. This effect has frequently been referred to as a common effect, or "C" effect, by extending Lerner's (1958) definition to asexually propagated material. It should be noted that the effect of cutting position, discussed previously, is another type of common effect.

Some studies have accounted for the variability among clonal cutting donors. For western hemlock, variation of rooting success among genetically identical donor plants was 6%, compared to 31% variation among genetically different clones selected randomly from a wild stand (Foster et al. 1984). Variation of this magnitude could partially obscure the selection of clones with strong inherent rooting capacities. Analogous variation in tamarack (*Larix laricina* Du Roi) was 11% among clonal cutting donors vs. 31% among random clones for one year,

but the "C" effect was insignificant for a second year (Farmer et al. 1992). The authors suggested that these effects would probably not be problematic, but could not be ignored.

Solutions

There are several approaches to handling variability within trees. Positional effects can be circumvented by selecting cuttings from specific locations within a tree. Selection for strong rooting capacity must be tempered with consideration for positional effects on growth rate and form. The latter will be discussed further in a subsequent section.

Heavy shearing to arrest maturation greatly reduces positional effects by limiting the origins of cutting production to a small, juvenile region. As previously discussed, these shoots are generally uniform in size and possess strong inherent rooting capacities.

Seasonal variability can also be accounted for or manipulated to some degree. For dormant cuttings, Struve (1980, 1982) developed a chill-unit accumulation model for eastern white pine based on a daily weighing method used for peaches (Richardson et al. 1974). Using needle fascicle cuttings from 54 six-year-old trees, he found that 58% of the variation could be accounted for by the model, whereas only 47% could be accounted for using calendar date as a predictor. Unfortunately, similar modeling of artificially chilled seedling top cuttings only explained a maximum of 5% of rooting variability. Since the test population was rather heterogeneous, the author suggested that accuracy might be improved if models were developed for individual clones.

Artificial exposure to constant cold has been used to simplify chilling of dormant cuttings. Miller et al. (1982) severed cuttings from five-year-old Fraser firs (*Abies fraseri* (Pursh) Poir.) in early October after shoots had received only 60 total hours below 5°C. They were assumed to be fully dormant, and were exposed to 0-12 weeks of dark storage at 4°C in sealed plastic bags before rooting. Rooting increased dramatically with only four weeks of chilling, but at least eight weeks of chilling was required to achieve 50% budbreak.

Wise et al. (1985a) used these results to postulate that mediocre rooting and budbreak observed with cuttings collected in mid-winter might be related to competition between the two processes for limited reserves. By chilling only four weeks to stimulate good rooting, the authors achieved up to 92% success (depending on crown position and subsequent treatments) with dormant Fraser fir cuttings severed from 14-year-old sheared trees. Cuttings chilled eight weeks generally rooted about 20% less. After further chilling so that all rooted cuttings received a total of 11 weeks at 4°C, new shoot growth for those initially chilled only four weeks was about twice as large as for those initially chilled longer. Thus the separation of rooting from budbreak in this fixed-growth species seems to have improved both responses.

To reduce variability among genetically identical cutting donors, Foster et al. (1984) recommend treating donor plants of the same clones uniformly. This would include establishing these plants from cuttings that were the same size and from the same position on the original tree. It should also include growing these plants under similar cultural conditions.

Summary

Due to the changing physiology of the donor plant, the physiological predisposition towards rooting varies strongly with the season in which cuttings are collected. Although winter cuttings of most conifers root relatively well, some have been rooted successfully at other times under certain conditions. Variability in rooting due to positional effects in tree-form donors can be reduced by selecting well-defined cuttings. Choosing tertiary axes from lower tree crowns may favor cuttings with a strong rooting capacity, but subsequent growth from these propagules may be undesirable. When rooted cuttings are further cultured to produce clonal cuttings, positional effects and differences in growing environments can contribute to significant non-genetic variability unless conditions are standardized.

INCOMPLETE TESTING AND SUCCESS OF CULTURAL ENHANCEMENTS

Problem

Propagators have tested a multitude of cultural treatments to improve rooting of cuttings, and these can be categorized and subdivided in many ways. In general, cultural enhancements can boost rooting success somewhat, but cannot entirely compensate

for poor rooting due to genetically inferior clones, maturation of cutting wood, or improper timing of collection. Despite the broad range of cultural factors that may influence rooting, relatively few good studies have been conducted on conifers; especially with juvenile cuttings. Still, a comprehensive review of these factors is covered elsewhere (Davis et al. 1988), and this discussion will highlight those that have shown promise for future enhancement of conifer rooting. For the purpose of this discussion, cultural enhancements are defined as manipulative inputs attempting to enhance yields of quality rooted cuttings that are made before, during, or after shoot severance. They will be covered in that order.

Pre-severance treatments.--The most important cultural manipulations conducted on donor plants are (1) treatments that influence the health and vigor of the plants, and (2) shearing treatments that affect yield and quality of juvenile cuttings from hedges.

Health and vigor.--Reports on procedures that produce good rooting from healthy, vigorous stock plants come primarily from horticultural literature, and contain evidence that is often anecdotal. Few of these reports deal with conifers important to commercial forestry. As an example, a symposium covering the effects of donor plants on propagation success (Read 1987) described or implied benefits from soil amendments, mulches, cautious use of herbicides, insect and pathogen controls, light exclusion, carbon dioxide enrichment, proper irradiance levels and photoperiods, and chemical growth regulators. Of the 137 references cited that dealt with specific species, only 10 involved coniferous species. Four of these ten were tissue culture studies, and most of the rest are covered somewhere in this document. The under-representation is more likely due to absence from the literature rather than editorial bias. Although these proceedings are neither an exhaustive review of donor plant conditioning, nor directly applicable to coniferous forest species, the above factors that they identified may be important for future examination.

There is further descriptive evidence, somewhat obscure, from successful horticultural nurseries. Iseli and Van Meter (1983) outlined the following procedures that produced healthy, economical cuttings of ornamental Norway spruce cultivars with enhanced rooting capacities:

"1. The nursery acquired stock plants which were containerized and maintained at optimum nutrition levels.

2. Weed, pest and disease vigilance was maintained and good control procedures established and adhered to on all containerized stock plants.

3. Use of herbicides as a weed control measure was suspended to ensure the optimum health and rootability of the plants. This requirement has been gradually relaxed with the limited use of Ronstar with no apparent deleterious effects.

It was felt that if the wood could be taken from super healthy and vigorous plants where disease and pest problems were minimal that good results would accrue; results vindicate that belief."

For forestry applications, Kleinschmit (1974) generalized that "rooting response can be increased markedly by systematic fertilizing" for juvenile Norway spruce. No data were presented.

There is scant direct evidence of specific benefits from proper fertilizing, watering, and light conditions. Enright (1959) applied supplementary 4-12-4 fertilizer and cottonseed meal to three-year-old, non-hedged nursery plants of three conifers and detected large increases in rooting percentages (Table 2). Although the fertilization effect was

Table 2. Rooting percentages of cuttings severed from three-year-old plants of three species, with and without supplementary fertilizer. For fertilized trees, 4-12-4 was incorporated into nursery beds at 560 kg/ha before planting, and top-dressed at 187 kg/ha during the first growing season. Cottonseed meal of undefined analysis was also incorporated and then top-dressed at 1121 kg/ha and 374 kg/ha, respectively. Means were calculated over three summer collection times from Enright (1959).

Supplementary fertilizer	Norway Spruce	Pitch pine	E. white pine
With	87	84	59
Without	16	1	14

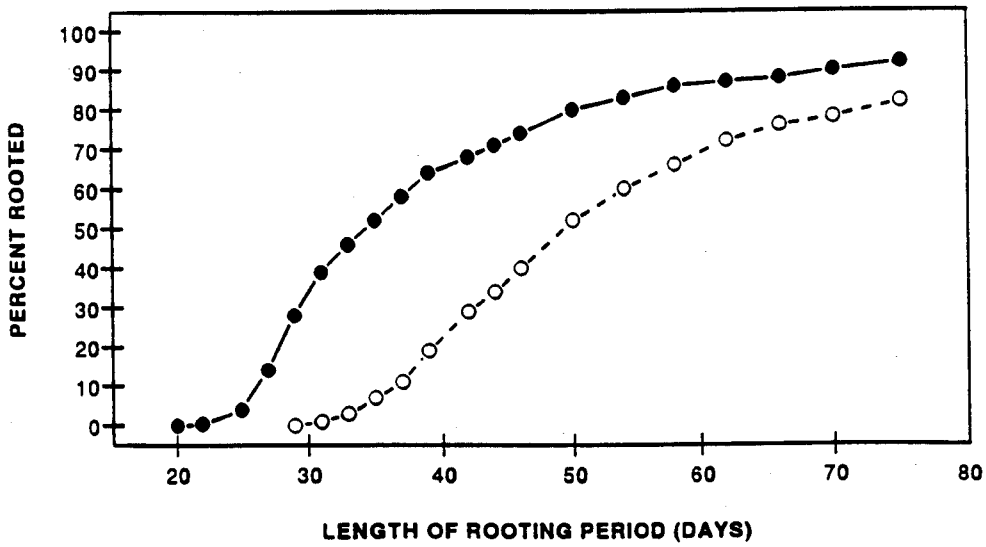

Figure 2.--Effect of irradiance on root formation of *Pinus sylvestris* hypocotyl cuttings. Seedlings were grown under 8 watts/m^2 (filled circles) or 40 watts/m^2 (open circles) for five weeks prior to rooting. Data from Hansen et al. (1978).

dramatic, extremely low responses from the non-fertilized but very juvenile plants indicates these donors were probably highly deficient. With juvenile Sitka spruce, a balanced and "moderate" nitrogen, phosphorus, and potassium regime applied in the growing season prior to cutting collection improved rooting over two other treatments (twice the moderate rate and a "high nitrogen" regime) (Mason and Keenleyside 1987). Although rooting from the moderate N-P-K treatment was 98%, compared to 90% from the other two treatments, it was not statistically better than the 95% achieved with a standard, low-fertility regime.

Light exclusion and water stress treatments have also influenced rooting in Sitka spruce (van den Driessche 1985). Containerized donor plants were propagated from hedges that were 15 years old from seed, and were 2 years old from cuttings when given 38% shade for 5 or 7 months, or not shaded. Dormant cuttings subsequently rooted at 53% from both shade treatments and only 34% from non-shaded controls. Although various forms of light exclusion have been shown to improve rooting in many angiosperms, this is one of the few examples that has shown a benefit to stem cuttings of a conifer. Two moisture levels were also superimposed on this study: (1) normal watering to maintain moist soil and (2) supplemental watering withheld about eight weeks in July and August. Only 33% of cuttings from stressed plants rooted while normal watering induced 53% success. Although this is good evidence for a water stress effect, the stress imposed was apparently quite extreme since 62% of non-shaded donor plants died before cuttings could be collected.

Donor plant light conditions have been explored further with a somewhat artificial system using Scots pine (Hansen et al. 1978, Hansen and Ernstsen 1982). For these studies seeds were germinated for one week, seedlings were grown five weeks under various light levels in growth chambers, and hypocotyl cuttings were then floated on aqueous solutions for rooting. Cuttings from seedlings grown at a relatively low light intensity, 8 watts/m^2, rooted more quickly and more frequently than those grown at 40 watts/m^2 (Fig. 2) (Hansen et al. 1978). This corresponded to lower contents of various carbohydrates, as seen in cuttings of some herbaceous species, and suggested that high light intensities induced supraoptimal carbohydrate contents for rooting.

Photoperiod was also important, indirectly, in a similar study (Hansen and Ernstsen 1982). Seedlings germinated throughout the year were grown in natural light at either natural photoperiods or 4-hour days. Constant short days nearly always boosted rooting, but mid-summer cuttings generally rooted poorly regardless of photoperiod. This implied that, in this system, the total radiant energy received during bright summer days was enough to stifle rooting even if exposure were short.

Cutting yields.--Beside improving rooting potential, cultural treatments can improve cutting yields. Although there are few reports comparing such treatments, New Zealand's Forest Research Institute has studied many manipulative techniques to produce good uniform cuttings from radiata pine

seedlings. One novel, intensive example (Fig. 3) involved growing seedlings 14 months, and then rather than hedging, pruning off succulent top growth and pinning the leaders horizontally to the ground to remove apical dominance (Menzies et al. 1985). Fascicular buds then expanded into upright cuttings, and were thinned during the season to improve individual quality. When dormant cuttings were collected, the shoot closest to the root system was retained intact on each plant. After these shoots grew early the next season, they were pruned and pinned to repeat the process. This procedure was developed primarily to multiply scarce quantities of genetically superior seed, but can potentially produce juvenile cuttings for several years and demonstrates the highly manipulable character of pines. Production systems will probably need to be tailored to meet the unique requirements of each of the desired species.

Summary

Cultural treatments that ensure the health and vigor of coniferous donor plants have been infrequently described and occasionally tested. A few studies have indicated benefits from controlled fertilization and light manipulation, but the full value of these treatments for enhancing rooting potential has yet to be realized. Similarly, managing juvenile plants by hedging or other techniques will be important for optimizing cutting production, but there is little public information on potential methods.

Treatments at severance.--The selection and removal of specific shoots from donors can influence rooting success. Although evidence conflicts, cutting length is more likely to influence cutting quality than to influence percent rooting success of juvenile shoots. For example, six-year-old Norway spruce shoots cut 5-20 cm long rooted at about 94% regardless of length (Radosta and Volna 1989). However, root collar diameter, the percentage of cuttings with branched root systems, and plant height after two years increased directly with original cutting length. Similarly, Fraser fir cuttings 10-26 cm long rooted around 78%, but the number of roots per rooted cutting increased with length (Miller 1982). Mean root length also increased with sizes up to 22 cm long. Conversely, rooting of shoots from hedges of a hybrid tropical pine (*Pinus caribaea* Morelet var. *hondurensis* Barr. and Golf. X *Pinus tecunumanii* (Schw.) Eguiluz and Perry) decreased as length increased from 10 to 49 cm (Haines et al. 1992). As with other species, roots per cutting and age two height increased with original cutting length.

Haines and others (1992) assessed several additional phenotypic characters to develop selection criteria for cuttings from hybrid tropical pine hedges. Shoots were collected in late summer so they were not dormant. Although genotype was the strongest predictor of rooting success, significant improvements in rooting and growth were predicted by selecting shoots with long primary needles, short but expanding secondary needles, and bases greater

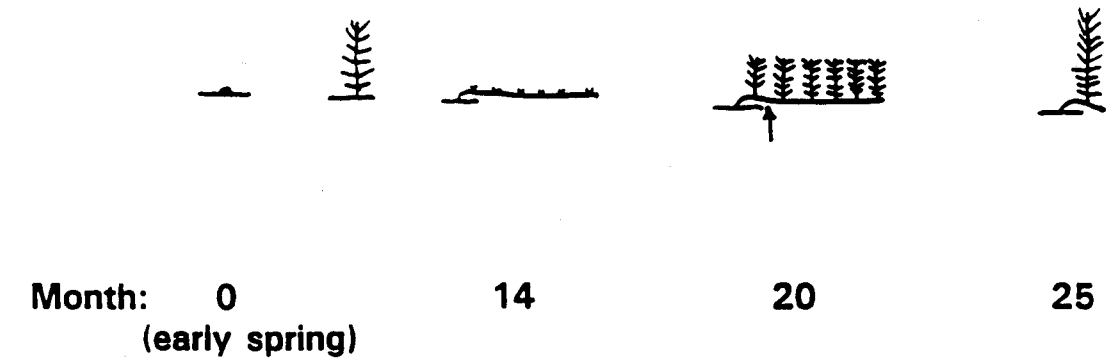

Figure 3.--Method for producing large cuttings from seedlings of radiata pine. Seeds are sown in early spring and seedlings are grown one year. In the spring of the second year, leaders are pinned horizontally to remove apical dominance, and emerging fascicular shoots are then thinned. Dormant cuttings are rooted the following fall. The main shoot is severed (arrow) to leave one fascicular shoot, and the pinning process is repeated the next spring. Adapted from Menzies et al. (1985).

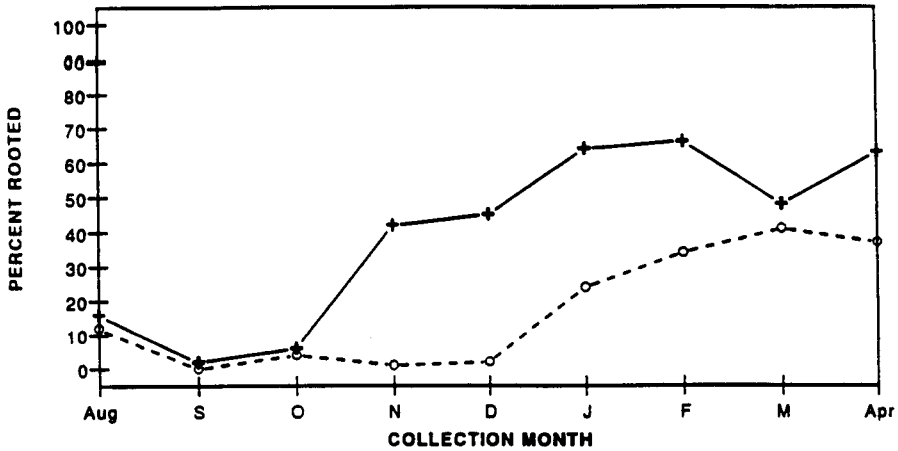

Figure 4.--Seasonal rooting averaged over two years for cuttings severed from 8- to 10-year-old sheared Douglas-fir trees and rooted with (+) or without (open circles) post-severance auxin treatment. Data from Roberts and Fuchigami (1973).

than 2 mm in diameter. Several other characters were less important. These criteria not only guide cutting severance, but indicate improvements can be made by precise timing of collection and hedge culture methods.

Post-severance treatments.--A wide array of manipulations can be imposed before cuttings are subjected to the rooting environment. Most have been reviewed extensively elsewhere (chapters 10-15, 17, 20 in Davis et al. 1988). Certain chemical treatments and handling procedures are most likely to be important for rooting conifer species. Due to the extensive nature of the topic, only major points will be briefly discussed.

Among the many chemicals applied to conifer cutting bases, the most beneficial are probably auxins, fungicides, and shoot growth retardants.

Haines and others (1992) assessed several additional phenotypic characters to develop selection

Auxins.--These hormones have been used to stimulate rooting for decades, but the practice involves many variables. Besides variable rates, there are several common chemical forms, and options for applying them as powders or liquids. For liquids, the solvent can be varied (usually an aqueous alcohol solution or pure water) and the treatment period can range from less than one second to several hours. Techniques and benefits have been summarized by Blazich (Chapter 10 in Davis et al. 1988).

The general benefit of auxin applications is to improve the quantity and quality of adventitious root systems on cuttings already somewhat predisposed to root. For example, a commercial auxin preparation improved rooting of cuttings from sheared 8- to 10-year-old Douglas-fir trees (Fig. 4), but cuttings collected late summer through mid-fall still rooted poorly (Roberts and Fuchigami 1973). Highest percentages were still obtained during the

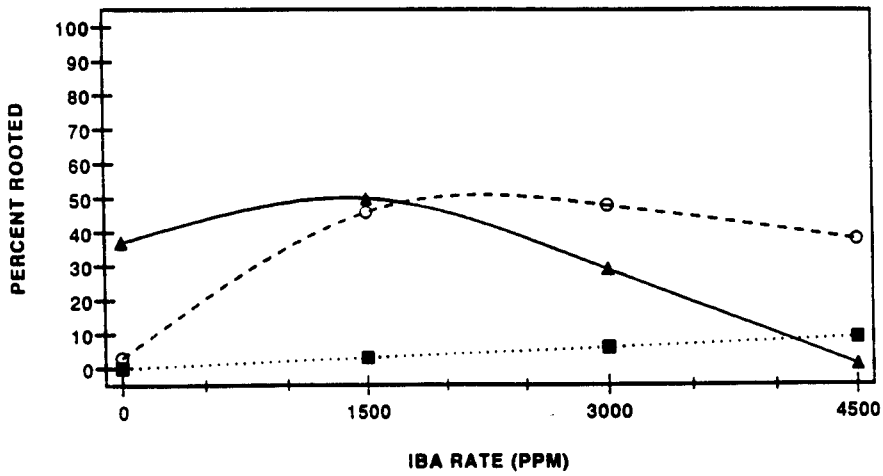

Figure 5.--Interaction of indole-3-butyric acid (IBA) concentrations, collection date, and shoot origin on rooting of Fraser fir stem cuttings collected June 23 (triangles), July 14 (circles), and August 4 (squares), from upper thirds of tree crowns. Redrawn from Wise et al. (1985b).

four months that had strongest inherent rooting capacity, but auxin hastened the transition to good winter rooting, perhaps by substituting for chilled buds. The major point, therefore, is that auxin applications have enhanced rooting, but they have not completely overcome poor physiological conditions due to maturation, branch order, cultural preconditioning, or other factors.

New synthetic auxins have shown promise in the last decade. Using jack pine (*Pinus banksiana* Lamb.) seedling tops, Haissig (1983) found that N-phenyl indolyl-3-butyramide (NP-IBA) increased percent rooted, number of roots, and longest root length over equimolar concentrations of a standard auxin, indole-3-butyric acid (IBA), and non-treated cuttings. Cuttings from non-sheared 7-year-old jack pine trees that were treated with NP-IBA tended to root slightly more frequently than those dipped in an IBA solution that was 37% stronger than the NP-IBA solution. Phenyl indole-3-thiobutyrate (P-ITB) and phenyl indole-3-butyrate (P-IBA) also were more efficient at stimulating rooting by some measures. Although these new auxins have improved rooting of a coniferous species, they have apparently remained largely untested with potential systems using juvenile donor plants.

Some studies have found no auxin benefit. Possible explanations could be that cuttings were not collected in the proper condition (as seen in the example in Fig. 4), were not subjected to an appropriate auxin treatment, were capable of rooting at high percentages regardless of auxin treatment, were collected from a biased genetic base, or combinations of these. Auxin response was strongly influenced by genotype of lodgepole pine (*Pinus contorta* Dougl.) cuttings from nine- and ten-year-old trees (Bowen et al. 1975). For one clone, rooting was less than 10% without auxin, increased to a maximum of about 75% near the mid-range of rates tested, and decreased to about 45% at the highest level. Two other clones did not respond to auxin; one rooted above 80% and another below 10% regardless of concentration. This clearly indicates that genotypes must be accounted for when refining auxin treatments, but similar work with juvenile shoots has not been widely published.

Interactions of timing and auxin rate have also been noted. For example, Fraser fir cuttings were severed from upper and lower crowns of sheared 14-year-old trees near the end of the annual growth flush and treated with relatively low auxin rates before rooting (Wise et al. 1985b). For upper crown cuttings, those severed in late June (before terminal buds were visible) rooted relatively well without auxin, but rooting percentage increased with a weak concentration and decreased markedly at higher rates (Fig. 5). Cuttings collected three weeks later only rooted well with auxin applications, yet rooted less well at the highest rate. After another three weeks, shoots were generally unresponsive although increasing auxin rates increased rooting slightly. Cuttings from the lower crown generally rooted in slightly higher percentages, as expected, but were more responsive to higher auxin concentrations. These results suggest that the internal complement of rooting promoters and inhibitors in softwood cuttings is highly dynamic, and that extensive testing may be necessary to find auxin treatments that enhance rooting by supplementing endogenous auxin.

In summary, auxin treatments have a strong record of enhancing rooting, but the wide array of possible treatment combinations makes exhaustive testing difficult. Testing is further complicated by interactions of auxin treatments with other important factors such as genotype and the physiology of shoots at specific times. Research to refine auxin treatments on shoots from juvenile conifer hedges is largely absent from the literature, but past trends indicate that gains of uncertain magnitude may be possible if other factors are properly accounted for.

Fungicides.--Applications of these chemicals are generally recommended for improving adventitious rooting (Hartmann and Kester 1983), and benefits have been shown for some conifers. Grigsby (1966) achieved 40% rooting with 25% captan plus 0.8% IBA applied as a powder to the bases of cuttings from six-year-old loblolly pines. This was three times greater than IBA alone. Cuttings from non-sheared 13-year-old eastern white pines responded more to fungicides than IBA levels when bases were dipped in treatment powders (Thielges and Hoitink 1972). No cuttings rooted without fungicide, regardless of IBA rate. Rooting percentage tended to increase with IBA rate when 5% benomyl was added, and results were generally best when 5% benomyl and 25% captan were both used. The fungicides were thought to indirectly enhance rooting by inhibiting pathogen attacks, rather than by directly stimulating root initiation. Although fungicide treatments have been beneficial for rooting somewhat mature conifers in the past, direct evidence is lacking for benefits in juvenile rooting systems.

Synthetic shoot growth retardants (SSGR).--These are used to improve the appearance of many ornamental crops, and have also been found to stimulate rooting in many cases. Their influences on rooting, and those of chemical inhibitors, have been recently reviewed by Davis and Sankhla (Chapter 13 in Davis et al. 1988). For conifers, one published report indicated that the SSGR paclobutrazol increased rooting in hypocotyl cuttings of loblolly pine (Rutter and Ingram 1988). The highest concentration (25 ppm) and longest soak period (24 hours) induced greatest rooting (93%). Water soak controls and a commercial auxin quick dip induced 73% rooting. Despite demonstrated benefits, breadth and magnitude of rooting stimulation have apparently not been sufficient to warrant commercial adaptation of SSGR applications.

Chemical combinations.--Significant work on the effects of combined chemical treatments on three pine species was summarized by Hare (1974). Independent experiments using cuttings from 1-year-old slash pine, 12-year-old Japanese black pine (*Pinus thunbergiana* Franco), and 13-year-old shortleaf pine (*Pinus echinata* Mill.) generally indicated that a specific powdered mixture of two auxins, the fungicide Captan, one SSGR, and sucrose applied to cutting bases was most effective. Juvenile slash pine cuttings collected four times throughout the year rooted at 100% with the complete or near-complete mixture.

The above formulation was referred to as "Hare's Powder" by later researchers. Unfortunately subsequent work with the preparation gave mixed results, and loblolly pine experiments provide an example. Hare's Powder improved rooting to 21%, from 6% with IBA alone, in cuttings from non-sheared 4-year-old loblolly pines (Greenwood et al. 1980). Pousujja (1981) reported increases ranging from 12% to 79% over IBA alone using one- to five-year-old non-sheared trees, but could not repeat high success rates in later experiments. For shoots from six-year-old hedges, those treated with the mixture always rooted similar to those that received 0.4% IBA regardless of season of cutting collection (Mahalovich et al. 1987). As with simple auxin treatments, it appears that complex mixtures of chemical rooting stimulants will have to be developed for specific production systems that account for juvenility, season of collection, and other influences.

Handling.--Handling treatments are the last to be discussed and are difficult to define and specify. In general, they consist of collection, storage and transportation techniques that seek to maintain or improve a strong rooting capacity in cuttings. Since cuttings are removed from their source of water, nutrients, and other growth factors, and can be temporarily subjected to fairly hostile environments, procedures should be adapted to maintain cuttings in the best health. However, references to handling procedures are infrequent, and those usually contain anecdotal evidence. For example, handling procedures have been important for rooting ornamental cultivars of Norway spruce:

> "All cuttings are taken early in the morning before heat of the day. They are collected in plastic tubs with a small amount of water in the base. When returned to the propagation house, cuttings are placed in a specially constructed bench which is endowed with a mist system to ensure the needles always possess moisture, thereby minimizing the possibility of shock. We have carried over for twenty-four hours cuttings in this environment with no apparent harm to rooting." (Iseli and van Meter 1983)

Grigsby (1971) compared two distinct sets of handling methods for rooting influences using shoots from non-sheared six-year-old loblolly pines. Dormant cuttings collected in early morning, always held vertically, cold stored for two days, and not recut before sticking rooted at 10%. Those collected in late morning, kept horizontal, and recut and stuck a few hours later without cold storage only rooted at 5%. The experiment was conducted with fairly mature material and was not designed to detect the most important handling factor(s), but gives some evidence that the way cuttings are processed can impact subsequent rooting. Hard evidence apparently has not been published for juvenile conifer rooting systems.

Solution and Summary

Some cultural manipulations made before, during, or after cutting severance have greatly affected rooting. Most have not been studied extensively, and few have been tried in juvenile production systems. Focused testing of these techniques within well-defined propagation programs may continue to enhance rooting, especially with selected families or individuals of recalcitrant species.

POOR FORM OF ROOTED CUTTINGS

Problem

Initial growth of rooted cuttings should be good in order for propagation to be fully successful. Reductions in shoot growth due to maturation of donor plants (Menzies and Klomp 1988, Dekker-Robertson and Kleinschmit 1991) and sub-optimal timing (Wise et al. 1985a) have already been mentioned. In addition, rooted cuttings of many conifers exhibit a branchlike, plagiotropic (*plagios*=oblique, *tropos*=turning) growth habit that can persist for a period of several years. For space considerations, this section will only discuss early plagiotropic growth.

One of the most interesting examples of the severity of the problem is provided by Black (1973). Douglas-fir cuttings from two clones, two and three years old, were planted normally in containers, planted in containers and staked vertically, or planted in the bottoms of containers and suspended in the air. After two years, the orientation of the leader growth within each clone was essentially the same for all treatments. The clone with the history of more severe plagiotropism grew about 35° from vertical and the other at about 14°. Therefore the severity of plagiotropic growth seems to be a strong genetic character that is not easily modified.

Severity of plagiotropism also interacts with maturation. Roulund (1975) demonstrated that three-year-old cuttings of Norway spruce grew more obliquely as the age of the donor plant increased. He concluded that cuttings should be selected from four-year-old or younger trees in order to obtain good rooting and growth for this species.

Plagiotropic growth has a rather curious physiological basis. It is generally known to be mediated by the formation of compression wood on what was originally the upper (adaxial) side of the branch, which is opposite of where it is usually formed. Starbuck (1979) also found greater cambial activity on adaxial sides of Douglas-fir cuttings, and increased short-term extension growth on adaxial sides of softwood cuttings. Although he also associated these processes with a gradient of apically-applied, radiolabeled auxin towards the adaxial sides, the gradient did not result from vertical reorientation. Biochemical mechanisms have not been elucidated further for conifer cuttings.

Although plagiotropic growth is primarily a problem with conifers that possess very regular branch orders, like spruces and firs, mild cases have been noted in the senior author's loblolly pine propagation program. Cuttings that were rooted in the greenhouse from greenhouse-grown hedges, transplanted into small tubes, and then returned to the greenhouse grew obliquely. Those from outdoor hedges that were transplanted into 4-liter containers and then returned outdoors grew upright. To determine whether light intensity, rather than root restriction, was mediating the response, cuttings from both origins were rooted in a shaded greenhouse and exposed to three light levels at wide spacing for nine months. Growth deviations from vertical increased linearly with decreasing light intensity for cuttings from both origins, but bending of greenhouse cuttings was exaggerated under denser shade. Adaxial compression wood actually began forming during the 12-week rooting period in most cuttings of both origins. This is evidence that pines are not immune to plagiotropism problems, but that propagators have some control over the severity of its expression.

Solutions

Other cultural manipulations to reduce plagiotropic growth have met with mixed success. Black (1973) was generally unable to enforce upright growth by staking rooted Douglas-fir cuttings from sheared trees, and the active bending actually broke stakes attached to several cuttings. With 15-year-old sheared Fraser fir trees, Wise (1986) reduced bending of one-year-old rooted cuttings by delaying original softwood shoot severance by about three weeks until lignification provided internal vertical support (33° v. 68°). A simple external wire support during rooting could be substituted for the delay (35° v. 68°).

Results have also been mixed with more juvenile material. Rooted primary branch tips from coast redwood (*Sequoia sempervirens* D. Don (Endl)) hedges were generally more upright than those from secondary branch tips (Power et al. 1988). However, seedling branch tips of the same orders grew more upright than corresponding cuttings from the hedges, which were seven years from seed. This suggests that shoot selection from hedges can be used to reduce plagiotropism in some species, but that hedge maturation may circumvent the improvement.

As a final example, Bentzer (1988) attempted to establish a "signal" within terminal buds that would cause shoots to grow upright. The objective was to restrain upper expanding shoots in an upright position so that their developing terminal buds would possess the "signal" for future upright growth. He used mesh cylinder bags on 3-year-old Norway spruce trees that had been serially propagated 3 times (14 years old from seed). He also pruned the leader from similar sets of trees (both with and without mesh bags), and selected cuttings by branch order. Unfortunately, restraining new growth upright actually increased the degree of plagiotropism, but it also improved rooting success (Table 3). It was concluded that just removing the leader improved cutting quality somewhat, especially if branch tips were collected only from the upper two whorls.

maintaining juvenility, correctly timing and choosing cuttings, imposing some beneficial cultural treatments, and minimizing early plagiotropic growth, there is great promise for even the most recalcitrant coniferous species. Enhancements can still be made in each area, and important interactions require further exploration. Cultural manipulations remain largely untested, especially with juvenile shoots, so additional attention to production, treatment, and handling of cuttings may also improve rooting capacity in some species. Regardless of the approach taken, a strong rooting capacity must still be captured by the correct rooting environment (see from these proceedings: Ford-Logan 1993).

To adapt rooting technology to many forest conifers, several key research questions still command attention for each species.

Table 3.--Rooting success and growth form (divergence from vertical) of Norway spruce cuttings from three-year-old plants that had leaders removed or upper growth forced vertically within cylindrical mesh bags prior to severance. Data from Bentzer (1988).

Treatment	Rooting	Plagiotropy
	percent[1]	score[2]
None (control)	90 b	2.1 b
Leader removed	92 b	2.0 b
Vertical bag	98 a	2.8 a
Leader removed plus vertical bag	98 a	2.7 a

[1]Means compared within columns by Duncan's multiple range test, 5% level.
[2]1=90°-82°, 2=82°-72°, 3=72°-58°, 4=58°-45°, 5=<45° from horizontal

Summary

Although plagiotropic growth has frustrated efforts to produce good-quality cuttings, it is certainly more problematic with some species than others. Treatments that retard maturation seem to also reduce the severity of plagiotropism, but do not completely arrest it. Correct timing, shoot selection, and clonal selection can foster a more upright initial growth habit, but physical restraints have not been generally successful.

GENERAL SUMMARY AND INTERPRETATION OF KEY RESEARCH ISSUES

Great progress has been made towards scaling the barriers to successful stem cutting propagation with conifers. By imposing some genetic selection,

How should rooted cuttings be used in tree improvement?--There are essentially two options: to deploy clones or to multiply scarce quantities of high-value seed. Historically, rooted cuttings have been used to perpetuate individual genotypes that possessed outstanding combinations of traits, primarily of horticultural crops. These genotypes could have been discovered by chance or through concerted breeding efforts. The population of genetically identical plants that would result from propagating one such genotype would be a clone.

The application of clonal techniques to forestry involves not only developing the methods to produce and root cuttings, but identifying the superior individuals to be propagated. In practice, clones are selected from field tests and then multiplied by cuttings into usable numbers for afforestation. Since maturation of selected trees would make them

difficult to propagate by the end of the test, a separate set of plants representing each clone in the test is maintained in the juvenile phase throughout the test. This option can consequently be expensive and time-consuming, but allows the exact genetic duplication of truly outstanding genotypes. For the future, it will also allow the perpetuation of individuals with genetically engineered traits without requiring the traits to be sexually transmissible.

The other option, often referred to as vegetative multiplication, generates juvenile plants from a limited supply of superior seed, and then roots cuttings from these seedlings. Methods for producing and rooting cuttings are required, but no additional testing or selection is needed. Consequently, the rooted cuttings are, genetically, only as good as the seeds that are multiplied by these methods. On the other hand, maturation is not a major problem since individual hedges are only retained to produce cuttings for only one to a few years. These techniques are often used with species that do not readily produce adequate quantities of seed. Alternately, they are often coupled with breeding programs that identify superior families at frequent intervals, and that want to deploy large numbers of trees of these families with a minimal lag time. In a recent world survey of cutting propagation in commercial forestry, it was concluded that "the primary use of rooted cutting technology is for bulk production of genetically improved half or full-sib families" (Ritchie 1991).

The choice of use depends primarily on the goals of each specific tree improvement program and its breeding component. Complicated or expensive methods may be necessary to clonally propagate some highly recalcitrant species. Consequently, the application of rooted cutting technology to these species may be limited to the simpler process of vegetatively multiplying seed.

How should production systems be developed?-- Focused research will be needed to develop unique production systems for each species. Since juvenile shoots have historically been critical to success, each system most likely will be based on maintaining juvenile donor plants through shearing or serial propagation. In addition, systems must work with identified sources of variability to generate cuttings with strong inherent rooting capacities. For example, cutting production should be timed so that juvenile shoots will be severed and rooted during periods of proven success. Promising seedling populations of known pedigree will doubtless be chosen as the starting material for vegetative multiplication, and for the development of clones. Additional selection can be used to perpetuate the best-rooting genotypes, or to cull those that root most poorly. However, this must be balanced against possible reductions in gains of economic importance (e.g. volume growth).

Cultural manipulations have often enhanced rooting, and tested treatments should be integrated into cutting production systems. Donor plants must be grown for the clear purpose of generating maximum quantities of cuttings with high rooting capacities. Therefore, maintenance of good health will be essential, and development of cultural treatments to ensure this through specific manipulations of water, fertilizer, light, and competition control may often be warranted. Cutting wood should be collected using defined size standards since size will affect the quality of adventitious root systems, and consequently the uniformity and repeatability of cutting crops. Chemical treatments applied after severance have frequently boosted rates of successful rooting or of root system quality under certain conditions. However, variability of response among and within species, and the wide array of potential chemicals, concentrations, and application methods will make exhaustive testing difficult. Regardless, strong reliance on these post-severance techniques is not warranted since they historically have not completely compensated for cutting wood that was not already somewhat predisposed towards rooting. It is most likely that general systems developed for species will eventually be adapted to specific clones and climates to further refine rooting success.

Rooting of cuttings has historically been labor-intensive. Horticultural crops have commanded relatively high prices to compensate for high labor costs per plant. Cutting collection, preparation, and rooting have generally not been automated because of the complexity of the tasks required, and the diversity of horticultural crops and producers. For forestry, there will be a strong economic incentive to minimize labor costs per plant due to the prevalence of low-cost seedlings. Fortunately, the large numbers of cuttings required to satisfy planting needs, and the relatively few nurseries that have historically produced forest tree seedlings, will help justify research expenditures directed towards automation. Future systems for rooting conifer cuttings are likely to draw upon existing and emerging technologies in materials handling,

mechanical sensing, robotics, and artificial intelligence.

How can we circumvent maturation?--Maturation has long been a major barrier to rooting, and successful propagation systems have been built around the maintenance of juvenile donor plants. In addition to enhanced rooting potential, juvenile phase shoots have generally been more uniform in size, and have produced plants that are largely similar to seedlings in form and growth rate. Unfortunately, there is evidence that procedures to maintain juvenility are not completely successful. Advanced research with Norway spruce clones has indicated that serial rooting of donor plants will not completely arrest maturation (Dekker-Robertson and Kleinschmit 1991). If it is assumed that long-term hedging is similarly insufficient, propagation of specific conifer genotypes as clones will not be possible for an indefinite period of time. However, for vegetatively multiplied radiata pine, the slight maturation that has occurred on hedges may actually improve the quality of sawtimber harvested from the rooted cuttings (Menzies and Klomp 1988, Arnold 1990).

With current technology, it takes many years to develop clones for afforestation. Genotypes must be field tested while juvenile material of the same genotypes is maintained separately. After superior clones are selected, it often takes one or more years to multiply these genotypes into large numbers of donor plants that will produce sufficient quantities of rooted cuttings for deployment. If maturation of genotypes proceeds slowly regardless of repropagation or shearing, testing and multiplication may leave little time for clones to be effectively utilized in planting. For Norway spruce, it was estimated that clones would be adequately juvenile for only 10 years following 10-15 years of testing and multiplication (Dekker-Robertson and Kleinschmit 1991). Burdon (1991) stated that the feasibility of developing clonal forestry techniques for radiata pine "is still dominated by maturation."

Additional research must make the development of clones more efficient. Ideally, methods to completely rejuvenate mature genotypes would eliminate the need for maintaining juvenile plants during clonal tests and, perhaps more important, might allow clonal tests to be composed of superior individuals selected from plantations or seedling progeny tests. Complete rejuvenation would also theoretically extend the useful lives of conifer clones for an indefinite period of time. Although rejuvenation techniques have been summarized (Hackett 1985), none have been complete enough to have been adapted to the testing and multiplication of clones. New rejuvenation techniques that are partially successful might be effective if coupled with hedging or serial propagation. Alternative methods to store genotypes during clonal tests, such as in vitro cold storage, may also retard maturation.

Development of procedures to hasten testing and multiplication of clones will also be important. Reliable early selection techniques, through conventional means or molecular biology, could shorten or eliminate clonal tests. Rapid multiplication procedures that may not be economically feasible for direct afforestation, like micropropagation or somatic embryogenesis, might instead be employed to multiply the number of donor plants for the production of field cuttings. In general, the creative synthesis of new and existing techniques should ultimately be able to extend the useful lives of conifer clones.

Is the technology economically feasible?--Commercial application of rooted cutting technology must be cost effective. The economic benefits of asexual propagules must surpass their added costs, and both of these elements require extensive investigation. If clones are to be deployed as rooted cuttings, the superiority and uniformity of selected genotypes must be quantifiable and repeatable. Selecting and deploying clones that are substantially superior to improved seedlings will be difficult because the process is complicated by maturation during the testing and multiplication phases, as discussed above. In recalcitrant species, intense selection for clones with strong rooting capacities may restrict potential growth gains, or require expensive clonal tests with large populations.

Assuming that the superiority and longevity of clones can be demonstrated, and that integrated propagation procedures can be developed, will the costs be justified? The question must obviously be addressed to specific production systems in specific climates. One example that compared radiata pine planting stock of equal genetic quality estimated that rooted cuttings from juvenile donor plants would cost 40-130% more to produce than seedlings (Menzies et al. 1985). To develop superior clones, costs incurred during clonal testing and multiplication would have to be added, but these would have to be weighed against the greater productivity of the clones. Although the question is simple to ask, the complex array of specific costs and benefits will make it difficult to answer.

Despite the immensity of some of the biological barriers to propagation, the relatively slow rate at which they have been solved through widely dispersed studies, and the research questions that remain, some conifers are being propagated on a large scale. To close, an example will demonstrate how solutions to the various problem areas were assembled into a commercial system for rooting conifer cuttings in the southeastern United States.

EXAMPLE OF COMBINED SOLUTIONS WITH LOBLOLLY PINE: INTERNATIONAL FOREST SEED COMPANY'S PROPAGATION PROGRAM 1988-1991

This program emphasized the production of clonal loblolly pine cuttings that were resistant to fusiform rust (*Cronartium quercum* (berk.) miyabe ex Shirai F. dp. *fusiforme*) and that produced good wood volumes (Foster and Shaw 1987). Seedlings resulting from controlled pollinations were exposed to rust screening methods at the U.S. Forest Service Rust Resistance Screening Center in Asheville, North Carolina (Anderson et al. 1983). Rust-free survivors at least six months old were then planted and maintained in hedge rows. Shearing techniques encouraged the development of many succulent juvenile shoots. Greenhouse rooting and field growth were assessed for cuttings from each hedge, and subsequent selections resulted in the establishment of a cutting orchard. The latter produced high quality cuttings that were rooted for planting stock.

One key to this clonal propagation program was the identification of donor plants that would repeatedly produce easily rooted cuttings. This required several propagation trials for each donor plant over a 12- to 18-month period. Later, genotypes were retested periodically to identify reductions in rooting capacity. A 2.8 ha cutting orchard resulted from these tests, and was composed of hedges of varying age classes. It provided an excellent opportunity to observe the effects of cultural regimes and shearing on several age classes of donor plants. Drip irrigation was used to provide accurate fertilization and consequently to ensure good health of hedges. Best hedge height was about 15-18 cm.

The rooting potential of genotypes identified as good rooters was enhanced by shearing; however, clones that rooted poorly were not significantly influenced by shearing (T. D. Caldwell, unpublished data). Further attempts were made to arrest maturation or rejuvenate older stock possessing desirable traits by serially propagating clones. The success of this technique has not yet been confirmed with mature stock. Two- to three-year-old stock plants were serially propagated to increase the numbers of desired clones. In most cases, the good rooters continued to express high rooting percentages. Unfortunately, genotypes expressing poor rooting were not retained for comparison since the commercial objective was to screen for good rooting clones. Consequently, the efficacy of serial propagation to enhance rooting ability of poor-rooting clones is unknown.

Although the orchard was capable of producing cuttings at least three times each year, the actual harvest only occurred in late fall. The decision to root only one cycle per year was made to circumvent problems with harsh weather and seasonal rooting variability. The extreme temperatures in the summer months adversely affected the vigor of the cuttings during harvest and propagation, which resulted in significantly lower rooting percentages (T. D. Caldwell, unpublished data). Earlier trials had indicated that the fall and winter collections were more likely to provide easily-rooted cuttings. Cuttings were therefore harvested by hand after becoming dormant. The actual dates of collection were usually after 15 November and before 10 December. Cuttings with terminal buds were preferred, but proximal shoot segments without terminal buds were also collected. These were pruned such that a lateral bud would eventually develop apical dominance.

Cuttings were placed upright in cold storage (3° C) for 24-36 hours. Before setting, the proximal end of each cutting was dipped in a proprietary version of Hare's powder (Hare 1974). Each was then set in a 97 ml cavity containing a soilless medium comprised of 60% perlite and 40% sphagnum peat moss. Planting density was 1000 cuttings per square meter.

Cuttings were then placed in a greenhouse for approximately 16 weeks where temperature, humidity, and light were closely monitored. Day temperatures were maintained at 25-26° C; night temperatures at 23-24° C. Winter air temperatures were increased using a radiant heating system located near the greenhouse roof. Relative humidity ranged from 85% to 92%. The variation in humidity corresponded roughly to day and night periods since

elevated day temperatures caused an increase in the moisture-holding capacity of the greenhouse air.

A traveling gantry boom irrigation system, positioned approximately 45 cm above the cuttings, provided intermittent mist. Utilizing normal waterline pressure of approximately 4 kg/cm^2 (60 psi) and nozzles that dispersed fine water droplets at a rate of 0.23 liter/min (0.06 gpm), a fog-like mist was deposited over the needle surfaces as the gantry boom traveled the length of the greenhouse. Manipulating the speed and frequency of travel regulated the amount of moisture deposited on the foliage. Uncallused cuttings required more frequent misting than cuttings that had callused or that had initiated root primordia. Operation of the gantry system was therefore regulated to match moisture applications to the physiological state of the cuttings. Two sources of artificial light extended natural day lengths to 16 hours during twelve of the sixteen weeks. Stationary 300-watt incandescent lamps were combined with similar lights attached to the misting boom to provide sufficient irradiance.

Approximately 10 weeks into propagation, temperatures and day lengths were gradually reduced to induce dormancy and to promote more root development with less shoot elongation. During weeks 11 and 12, the greenhouse would be returned to outside ambient temperatures. Day temperatures would therefore range from 5 to 12° C; night temperatures from 5 to 7° C. The cuttings were returned to natural day lengths by the end of week 12.

The rooted cuttings remained within the greenhouse until the following spring after all danger of a late freeze had passed. The rooted cuttings were usually moved outdoors after 15 April. Cuttings then received soluble 20-20-20 general purpose fertilizer at ten-day intervals through the summer. The last application occurred in mid- to late September when irrigations with clear water were substituted to lower nitrogen levels in preparation for dormancy. Fungicides and pesticides were applied as needed. The rooted stock was considered to be field ready by late fall, approximately 12 months from cutting harvest.

This rooting system was designed specifically to propagate one coniferous species. Many components, however, were similar to those in vegetative propagation programs found primarily within the ornamental nursery industry. It was critical to control humidification, temperature, nutrition, medium, and seasonal harvest timing. Many factors were interrelated and required balance to stimulate development of adventitious root systems. Two factors that caused loblolly pine to be difficult to propagate vegetatively were (1) the inevitable loss of the strong juvenile rooting capacity, which often resulted in dramatic reductions in rooting success, and (2) the resulting requirement for intensively selecting clones that maintained strong rooting capacities past the second or third shearing cycle (about 2 years of age).

Barriers to successful propagation were successfully scaled to institute this program. Since loblolly pine was, and will continue to be, an important species, selection within the species to provide outstanding growth, survival, and rooting capacity was important. By shearing the donor plant, maturation was retarded and many succulent, juvenile shoots were forced into growth. How long this technique can arrest maturation will probably be dictated by the species, and by the individual donors and their response to continuous hedging and other treatments. Collecting cuttings in the fall, when they had a relatively strong rooting capacity, improved crop success and consistency. Water and fertilizer applications enhanced productivity and ensured that cuttings on healthy hedges would have a strong capacity to form roots. A highly-controlled greenhouse provided the correct balance of environmental factors that allowed cuttings to root.

Currently, there are no large scale clonal propagation programs for loblolly pine. The costs of such a labor- and equipment-intensive program may continue to deter many organizations from pursuing the necessary technology. However, as the biological barriers to rooting are scaled, and logistical problems are resolved, clonal propagation should become a viable forest regeneration option for this and other coniferous species.

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ROOTED CUTTING MACROPROPAGATION OF HARDWOODS[1]

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Abstract.--Factors affecting regeneration from cuttings of hardwood tree species include (a) rejuvenation and conditioning of the stock plant, (b) environmental conditions and physiological status of the stock plant, (c) genetic differences in rooting ability, (d) seasonal timing of collection, (e) cutting size, node characteristics, and position on the stock plant, (f) treatments of cuttings, and (g) planting methods.These factors are discussed and illustrated with examples for hardwood species. A condensed table of recommendations for cutting macropropagation of 16 hardwood genera is included, and the current status of several operational programs is given. Management of within-clone variability is discussed.

INTRODUCTION

Macropropagation with shoot and/or root cuttings has been accomplished for many hardwood tree species. These cuttings can provide an economically viable alternative for artificial regeneration if (a) unrooted hardwood shoot cuttings can be planted directly in the field with high rooting success or (b) rooted cuttings can be mass produced in a nursery/greenhouse and outplanted to the field at an acceptable cost. Situation (a) can be met for some (but only a few) hardwood timber species, as are identified later. Situation (b) requires either cheap labor, highly mechanized nursery methods, rapid growth with short rotations (energywood, pulpwood), or high-valued products (fine furniture wood, urban trees, multiproduct agroforestry species, or horticultural nut and fruit species). Some species are so difficult to root that they will probably never be regenerated with cuttings.

We will first define some terms used throughout the paper and in the rooting literature. General factors affecting rooting success from shoot and root cuttings of hardwood tree species will then be reviewed and illustrated with examples. These factors include (a) rejuvenation and conditioning of the stock plant, (b) environmental conditions and physiological status of the stock plant, (c) genetic differences in rooting ability, (d) seasonal timing of collection, (e) cutting size, node characteristics, and position on the stock plant, (f) treatments of cuttings, and (g) planting methods. The third section of the paper provides specific recommendations for cutting macropropagation of 16 hardwood genera and summarizes the current status of several operational hardwood programs using cutting macropropagation in the United States. The final section discusses the need for control of within-clone variability in production programs, once the methods for getting high rooting success have been developed. Sources of within-clone variability that are discussed include (a) phenotypic (non-persistent) variation, (b) epigenetic

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variation, (c) genetic variation within clones, and (d) variation caused by pathogens. A suggested strategy of seven steps is given for management of cutting production programs to control these sources of within-clone variability.

TERMINOLOGY

The rooting literature is notorious for jargon. To assist the reader, several terms and concepts used in this paper are defined below. These definitions come from Hartmann et al. (1990, Chapters 1, 8, and 9) and from the Society of American Foresters' "Terminology of Forest Science, Technology, Practice, and Products" (SAF 1983). Additional definitions are provided at appropriate places throughout the paper.

Adventitious.--Adventitious refers to a plant part that develops outside the usual order of time and/or position (outside the normal order of plant development and ontogeny). **Adventitious buds (and shoots)** arise from any plant part (stem, leaf, or root) other than terminal, lateral or latent buds on stems. The origin of adventitious buds is usually in callus or lateral meristematic tissue, and these buds have no vascular connection with the pith. In contrast the terminal, lateral, and latent buds have a vascular connection with the pith, indicating that they originated from terminal meristematic tissue and were preformed in the normal order of plant development. The **latent buds** are dormant buds lying under the bark, and they begin to grow when dormancy is broken by wounding or by increased sunlight on the stem. **Adventitious roots** arise from any plant part (stem, leaf, or root) other than from terminal meristems in root tips. Adventitious buds and roots are initiated in response to some wounding and/or chemical stimulus. Adventitious roots can be either **preformed roots** (develop on stems while the stems are still attached to the parent plant) or **wound roots** (occur in response to a wounding effect, and can develop after the plant part is removed from the parent plant).

Cuttings.--A cutting is a segment or part (greater than a few cells or piece of tissue) that is cut from a living stem, limb, root, or leaf. It is used to produce a whole new plant through the development of adventitious roots and/or shoots. Stem or limb cuttings require only the development of adventitious roots, and these cuttings can be classified according to the degree of lignification (hard woodiness) of the tissue. **Softwood cuttings** (also called summerwood or greenwood cuttings) are those cut from the current year's tissue while the parent plant is actively growing. These cuttings are taken early in the growing season from near the growing tip, have little lignification, and are soft and fleshy with a high water content. **Hardwood cuttings** (also called dormant cuttings) are those collected during the dormant season of the year. They are lignified woody cuttings and may come from the most recent past growing season (usual) or from earlier growing seasons. **Semi-hardwood cuttings** (also called firm softwood cuttings) are taken late in the growing season from the current year's growth and are partially lignified. **Root cuttings** are usually taken from lignified woody roots and must initiate both a new shoot system (from an adventitious bud) and new adventitious roots. Sometimes these cuttings have adventitious shoots (root sprouts) already formed while the root was still attached to the parent plant. More commonly, the adventitious roots and shoots are formed after the root cutting is detached from the parent plant. In the present paper the word "cutting" usually means stem, limb, or sprout cuttings. A root cutting will be specified as "root cutting".

Sprouts.--A sprout (also called a shoot) is a young, slender, aerial outgrowth from a woody plant part (stem, limb, or root). Sprouts are often used as sources for cuttings, because they retain some juvenility and give cuttings with higher rooting success. **Coppice sprouts** (also called stump sprouts or stool shoots) are any shoots arising from adventitious or latent buds near the base of a woody plant that has been cut back (detopped). **Epicormic sprouts** (also called water sprouts) are shoots arising from adventitious or latent buds on the stem or on a limb of a woody plant. The development of these sprouts is usually in response to wounding or to an increase in sunlight reaching the stem.

Coppice sprouts are epicormic sprouts, but epicormic sprouts also include shoots further up the tree. **Root sprouts** (also called root suckers) are aerial shoots arising from adventitious buds on roots or rhizomes below ground.

Rooting ability.--Rooting ability (also called rooting capacity or rooting success) refers to the ease of adventitious root initiation on cuttings (and adventitious shoot initiation on root cuttings). This is typically measured by the percentage of cuttings which establish a root and shoot system and survive

for some defined period of time (such as to the end of the first growing season).

Juvenility and maturity.--Any tree in its life cycle goes through embryonic, juvenile, transitional, and mature (adult) phases of growth and development. The juvenile phase has a greater ability to develop adventitious roots and shoots than the mature phase, but the mature phase can produce flowers while the juvenile phase cannot. These are **epigenetic** changes, meaning that the differences result from changes in gene regulation rather than changes in the genetic makeup of the plant. Because the juvenile phase exhibits the higher rooting ability, that phase is preferred for macropropagation with cuttings. Therefore, an important topic in macropropagation is how to control juvenile-mature changes (the aging process). Possibilities include (a) use of the most juvenile part of the tree (the base of the stem) and (b) reversions of the aging process. This aging process involves both physiological and ontogenetic aging. **Physiological aging** is identified by reduced growth rate, change in type of branching, and exhaustion of nutrient content as the source plant gets older and larger in size (Hartmann et al. 1990, Struve and Lineberger 1988). It is not localized in the meristem and can be reversed by **renewal or invigoration** of shoots through pruning, nutrient additions, or stand density control. Invigorated shoots may root better than non-invigorated shoots, but they can also retain mature traits such as flowering (Hackett 1988). **Ontogenetic aging** refers to the epigenetic change from the juvenile to the mature phase. It is genetically programmed, localized in the meristem, and not related to exhaustion of nutrients. **Rejuvenation** represents the reversal of the ontogenetic aging process. Rejuvenated shoots will root easily, will usually not flower, and will exhibit morphologically juvenile traits, such as juvenile leaf forms.

FACTORS AFFECTING ROOTING OF CUTTINGS FROM HARDWOOD TREE SPECIES

Age and Conditioning of the Source Plant

Rooting ability of shoot cuttings decreases with increasing tree age for hardwood tree species. For some of the eucalypts rooting success declines so rapidly that cuttings collected from one-meter-tall seedlings will not root (Hartney 1980). Typically, however, once successfully rooted first-generation cuttings of mature trees are obtained, they will produce rejuvenated cuttings of increased rooting capacity. This has been reported for *Eucalyptus tereticornis* Sm. *x E. camaldulensis* Dehn. by Franclet (1983). The problem is getting the rooted first-generation cuttings from the mature source plant.

Several methods listed by Hackett (1988) for improving rooting from mature plants include: (a) severe pruning or hedging, (b) grafting mature scions on juvenile rootstocks, (c) adventitious bud initiation, and (d) in vitro propagation. The first three are discussed here, and the fourth is included in the paper by Scott Merkle (1993) at this symposium.

Severe Pruning or Hedging.--Many hardwood species can produce coppice sprouts from the base of a tree. These sprouts often have high rooting ability that is comparable to cuttings from seedlings, since they originate from a juvenile part of the tree. This characteristic has been the basis for a number of operational macropropagation programs throughout the world, where stool beds or cutting orchards are established from selected trees and repeatedly coppiced or severely pruned to produce cutting material. Franclet (1983) believes that this form of hedging results in selection of meristems which remain juvenile and stabilized at that maturation level, rather than true rejuvenation. In addition to maintaining juvenility, pruning or hedging of stock plants controls plant shape for ease of stock management and cutting collection, controls the timing of flushes, and increases cutting production (Scott 1987).

There may be instances where severe pruning or coppicing is not an option for the production of epicormic shoots (e.g. the selected tree is needed for breeding purposes). One alternative method is to partially girdle selected trees to temporarily relieve apical dominance and allow coppice sprouts to develop. This technique has been successfully employed to initially propagate *Quercus rubra* L. (Harmer 1988), yellow-poplar (*Liriodendron tuliperifera* L.) (McAlpine and Kormanik 1972), sweetgum (*Liquidambar styraciflua* L.) (Kormanik and Brown 1973) and several *Eucalyptus* L'Her. species (Hartney 1980). Juvenile sprout material for macropropagation has also been obtained through the application of cytokinins to the base of trees to induce latent bud break. Mazalewski and Hackett (1979) reported that the application of cytokinins as a lanolin paste or water-ethanol solution to the base of *Eucalyptus ficifolia* F. Muell. trees was successful

in inducing bud break in the lignotubers and in the upper trunk region. However, only shoots obtained from the lignotubers possessed high rooting ability. They concluded that the position on the trunk from which epicormic shoots were obtained reflected rooting ability of the original seedling nodes from which they were derived.

Grafting Mature Scions on Juvenile Rootstocks.--A second alternative (when the selected tree must be retained for breeding purposes) is the grafting of mature scions onto seedlings. This is followed by either (a) hedging or partially girdling the grafted tree after a few years to stimulate epicormic sprouts or (b) repeated grafting in serial fashion at short intervals on successive seedlings. The procedure has been an effective method of obtaining cuttings with increased rooting ability for several species. Farmer and Besemann (1974) combined grafting and severe pruning of selected black cherry (*Prunus serotina* Ehrh.) to substantially improve the rooting of softwood cuttings propagated under mist. However, there were no non-pruned controls to determine if grafting or pruning effects had improved rooting. Paton et al. (1981) proposed that the improved rooting of cuttings from mature scions of *Eucalyptus grandis* Hill ex Maiden. grafted onto juvenile seedlings is a result of a closer association between the shoot tip and the base of the stem and/or roots ("root-shoot gradient"). They compared rooting ability and levels of the growth regulator G (a compound which has been shown to inhibit rooting of *E. grandis* at high concentrations and promote rooting at lower levels) for (a) cuttings taken from the distal and basal positions on adult trees and (b) scions of adult trees grafted onto seedlings. In each case they found that epicormic shoots at the basal position had lower G concentrations and higher rooting percentages. Franclet (1983) and Hackett (1988) believe that *E. grandis* cuttings taken from epicormics at the base of mature scions grafted on seedlings have been rejuvenated. Repeated grafting in serial fashion is also thought to rejuvenate cuttings when used for *Eucalyptus x trabutii* (= *E. botryoides* Sm. *x E. camaldulensis*) (Hartmann et al. 1990) and *E. camaldulensis* (Franclet 1983).

Adventitious Bud Initiation.--Adventitious shoots on roots or sphaeroblasts of *Populus tremuloides* Michx., *Ulmus* L. species, and apple cultivars produce rejuvenated cuttings of high rooting success (Hackett 1988). Aspen tree improvement programs rely on the production of these shoots from root segments for macropropagation. The techniques described by Benson and Schwalbach (1970) consist of collecting root segments from selected trees, culturing the roots under greenhouse conditions to produce adventitious shoots, and then rooting the softwood sprouts. Success rates of from 70 to 90 percent are obtained with most aspens. A similar system has been used for sweetgum (Farmer 1966). Stimulation of adventitious buds for coppice and epicormic shoots by severe pruning/hedging and by grafting have been discussed above.

Environmental Conditions & Physiological Status of the Stock Plant

Macropropagation of selected trees under forest conditions severely restricts the capabilities of stock plant environmental control. If large numbers of cuttings are required, it is best to establish stock plants in cutting production orchards (stool beds). Doing so provides a number of advantages: (a) tree health is known, (b) management is easier, since the propagator controls plant nutrition, weed control, pruning, pest and disease control, and all aspects of plant manipulation; and (c) tree growth is more uniform, since cuttings root more evenly (Scott 1987). Most operational rooted cutting programs have established cutting production areas for these reasons.

Nutritional status of the stock plant, and particularly the carbohydrate-to-nitrogen ratio (C/N), is frequently discussed as an important factor for root initiation. High C/N has been shown to improve rooting for a number of species (Moe and Anderson 1988). One method of obtaining this high ratio is to reduce N supply to stock plants (Hartmann et al. 1990). In a study examining clonal differences in rooting of sycamore coppice sprouts, Cunningham (1986) found significant differences in rooting between clones grouped by the progeny test block in which the stock plants were located. These blocks also differed in soil N content. Rooting percentages for the four blocks were 68, 59, 54, and 35, and these corresponded to soil N levels (ppm) of 473, 705, 650 and 822, respectively. While no firm conclusions could be drawn regarding the effects of soil N levels on rooting due to a lack of common genotypes among treatments, clones from the progeny test block with low soil N rooted significantly better than those from the high soil N block.

Carbohydrate status of cuttings can be increased by girdling shoots prior to severance. The technique

developed by Hare (1976a, 1976b, 1977) for a number of species involves removing a small strip of bark around the stem to allow for the accumulation of carbohydrates as well as other translocated substances at the distal end of the girdle. After six to eight weeks the girdled shoot is removed from the tree and used for propagation. In nearly all cases, Hare has found that cuttings from girdled shoots root significantly better than their nongirdled controls.

Cutting macropropagation is also influenced by the light conditions under which stock plants are grown. Increasing light irradiance in the greenhouse may increase the carbohydrate content of the cuttings. However, light reduction has often been beneficial for rooting. This may be because flowering and vegetative growth is inhibited to release more carbohydrate for rooting (Hartmann et al. 1990). Reducing stock plant irradiance through shading and reduction in photoperiod is a common practice to enhance rooting (Bachelard and Stowe 1963, Hansen 1987). Total exclusion of light from newly emerging shoots for a period of time prior to propagation (etiolation) has proven effective in promoting rooting for a number of species (Hartmann et al. 1990). A slight modification of this procedure, where light is excluded from the cutting base prior to propagation (banding), may also improve rooting. The girdling procedures of Hare (1976a, 1976b, 1977) would be considered banding, since the girdled portion of the stem is covered with aluminum foil. Mixed results have been obtained in etiolation trials with forest species. Kormanik and Brown (1973) found that etiolated shoots of sweetgum and yellow-poplar rooted poorly while Farmer and Hall (1973) found that black walnut (*Juglans nigra* L.) etiolated shoots rooted well. Additional explanations for the effects of etiolation and banding on rooting include changes in stem tissue anatomy (less lignification, decreased cell wall thickness and reduced cell differentiation) and increased tissue sensitivity to exogenously applied auxin (Bassuk and Maynard 1987).

Genetic Differences in Rooting Ability

There is a wide range in rooting ability for species, geographic origin, and genotype just as there is for other more commonly measured traits of forest trees. One of the first reports of tree-to-tree variation in rooting was for red maple (Snow 1939). Since then genetic variation has been reported for a large number of species (Haissig and Riemenschnieder 1988). The selection of good genotypes can dramatically improve overall rooting percentages, provided rooting ability is under genetic control. For example, eucalypt programs are finding that clonal means for rooting percentages of genotypes selected for growth characteristics can range from 0 to 100 percent (Campinhos and Ikemori 1980).

Studies evaluating the level of genetic control have found broad-sense heritabilities on a clone mean basis to be fairly high, but few estimates of additive and non-additive genetic variance are available. Wilcox and Farmer (1968) estimated broad-sense heritabilities for eastern cottonwood (*Populus deltoides* Bartr. ex Marsh.) to be from 0.32 to 0.89 for a number of rooting traits. With a different population of eastern cottonwood, Ying and Bagley (1977) estimated broad-sense heritabilities for numbers of roots initiated to be 0.8 and 0.86, and they detected significant levels of additive variance. Rooting abilities for both sycamore and sweetgum have comparable broad-sense heritability estimates to those for eastern cottonwood, but estimates for additive variance were nearly zero (Cunningham 1986, Cunningham 1989). One common limitation to each of these studies is the use of open-pollinated families, where no direct estimates of non-additive variances can be obtained. In addition, the work with sycamore and sweetgum used only a limited number of families and clones within families, so the precision of estimates was low.

Seasonal Timing of Collection

The time of year in which cuttings are collected can have a dramatic effect on rooting and may be the key to success for many species. Easily rooted species, such as those possessing preformed roots, can generally be rooted all year and are operationally rooted using hardwood cuttings. In contrast, many species can only be rooted using softwood cuttings. Sweetgum is a good example of such a species. Cuttings from either tree crowns or coppice shoots root fairly well when collected during the growing season; however, dormant material will not root (Cunningham 1989) (Table 1). Hardwood cuttings can generally be collected any time during dormancy provided they are outplanted at the proper time, and proper storage procedures have been followed (Fege 1983, McKnight 1970). However, if cuttings are set immediately following collection, some differences may be seen. Kefeli and Turetskaya (1965) found that willow cuttings collected in the fall rooted poorly in comparison to spring collections. But, chilling or removal of buds

Table 1.--Influence of cutting type and location on sweetgum rooting success

Cutting position	Cutting Type: Softwood	Cutting Type: Hardwood
	% rooted	
Coppice sprout	84	0
Tree crown	52	1

from the fall-collected cuttings improved rooting success to levels equal to those of spring-collected cuttings. They attributed the poor rooting to the accumulation of flavonoid compounds in the buds during the fall which neutralize the stimulating effects of auxin. Over the winter months the concentrations of these inhibitors decline to levels where root initiation can proceed in the spring.

The time of collection during the growing season is very important to rooting success of softwood cuttings. For some species rooting success is best when cuttings are collected early in the season, whereas for others a semi-hardwood cutting collected in late summer is best (Dirr and Heuser 1987, Hartmann et al. 1990). For deciduous species collection time during the growing season will also impact the length of time for root and shoot growth to occur prior to the onset of dormancy. Cunningham (1989) found that cuttings derived from early collections of sweetgum coppice sprouts, while not having higher rooting percentages, were significantly larger at time of planting than later collections (Table 2). Increasing lignification and concentrations of root-inhibiting growth regulators in later collections is a problem for eucalypt species (McComb and Wroth 1986, Paton et al. 1981).

Type of Cutting Material Collected

Cutting Position on Tree or Sprout.--Hardwood cuttings from the basal portion of sprouts tend to root and grow better than cuttings from more distal portions of the stem. This has been reported by several authors for sycamore (Briscoe 1963, Land 1983, Nelson and Martindale 1957). Nelson and Martindale (1957) and Land (1983) separated potentially confounding effects of cutting diameter in their analyses and demonstrated that cutting position affected first-year height and seventh-year dbh and volume, respectively.

Findings similar to those for sycamore have also been reported with *Populus* L. species (Hansen and Tolsted 1981, Schroeder and Walker 1991). Differences in performance based on cutting position have been attributed to the number of preformed root primordia and carbohydrate levels. Distal positions on poplar shoots tend to have fewer root primordia than basal cuttings (Smith and Wareing 1972a). Although they have higher concentrations of carbohydrates, the total amount of carbohydrate in the stem is less for shoot tip cuttings than for cuttings from the sprout base (Fege and Brown 1984).

Table 2.--Influence of growing season collection date on rooting and growth of sweetgum cuttings from coppice sprouts

	Collection Date	Rooting Success		Cutting Height		Root-collar Diameter		Root Growth[1]
		%		cm		mm		%
	June 7	88a[2]		15.9a		4.2a		65a
June 21	91 a		14.0 b		3.9 b		51 b	
	July 7	91 a		11.9 c		3.5 c		37 c
	July 19	89 a		11.8 c		3.1 d		10 d

[1] Root growth was measured as the percentage of rooted cuttings with sufficient root development to form a complete plug when removed from the container.

[2] Collection date means followed by the same letter within a column are not significantly different at the 5 percent level.

For softwood cuttings the situation is reversed. Cuttings taken from the shoot tip generally root better than those from farther down the shoot (Leakey and Coutts 1989, Cunningham 1989). The better response of apical softwood cuttings has been attributed to higher concentrations of root promoting chemicals at the shoot apex and a lower degree of stem lignification (Hartmann et al. 1990).

Cutting Size and Node Characteristics.-- Softwood cuttings are typically short (8 to 15 cm long), and consist of the current year's growth after some degree of lignification has occurred. Leaves are always attached, as rooting percentages will drop dramatically if these leaves are removed (Bilan 1974, Bachelard and Stowe 1963, Geary and Harding 1984). Macropropagation programs with eucalypt species generally consist of cuttings with two to four nodes, depending on internode length. Eucalypt cuttings for the Aracruz program in Brazil contain two nodes and four leaves to allow for some leaf senescence (Campinhos and Ikemori 1980). However, Geary and Harding (1984) reported that while *Eucalyptus camaldulensis* cutting yields from stock plants were doubled by using shorter cuttings with two nodes, rooting success of the two-node cuttings was significantly lower and not sufficient to produce more rooted cuttings than the four-node propagules.

Hardwood cuttings usually are collected from one-year-old sprouts during the dormant season. For *Populus deltoides* in the South, the average cutting length used for field planting is 50 cm. Longer cuttings might be used on drier sites (McKnight 1970), and shorter cuttings might be used for nursery propagation (Cunningham 1986). In other regions of the United States, hardwood cuttings are usually shorter, and average 20 cm length (Fege 1983). Diameters of hardwood cuttings typically range from 13 to 25 mm. Cuttings larger than this will root, but they are more difficult to handle (Fege 1983, McKnight 1970). The survival and growth of field-planted *Salix alba* L. cuttings increase markedly as cuttings increase in size up to a diameter of 19 mm and length of 20 cm (Burgess et al. 1990). Rooting success declines for sycamore cuttings with less than a 15-mm diameter, but is equally good for 15- to 22-mm-diameter cuttings (Land et al. 1991) (Table 3). Improvements in survival and growth with increasing cutting diameter may differ by clone. In a study comparing the effects of cutting diameter on growth of three *Populus* clones, Dickmann et al. (1980) found that cuttings from 6- to 19-mm diameter survived and grew equally well for 2 of the 3 clones, but declined for cuttings below 10-mm diameter for the third clone.

Table 3.--Influence of cutting diameter, number of nodes, and proximity of bottom node to base of cutting on rooting success of 25-mm hardwood sycamore cuttings

Basal Diameter		No. Nodes Below Ground		Length from Cutting Base to Lowest Node	
Diameter	Rooted	Number	Rooted	Length	Rooted
mm	%	No.	%	cm	%
<=12	10	0	0	0-2	53
13-14	17	1	27	3-4	32
15-16	42	2	38	5-6	34
17-18	56	3	36	7-8	25
19-20	52			9-10	33
21-22	50			>10	14

Hardwood cuttings should consist of several nodes, and location of the nodes is important. Land et al. (1991) found for sycamore that having at least two nodes below ground and a node within two cm of the cut base resulted in best rooting (Table 3). Having a node near the top of the cutting, and having the correct type of buds present at the nodes,

may be critical for rooting of *Populus* species. Radwan et al. (1987) found that the best-growing cottonwood cuttings were those possessing axillary buds, rather than dead buds or spent buds (nodes where a branch had elongated). The authors hypothesized that the slower growth exhibited by cuttings with spent or dead buds was a result of the time lag involved in the development of suppressed or adventitious buds at the nodes.

Treatment of Cuttings

Hardwood cuttings are often stored prior to planting as a convenience to nursery managers. This storage may or may not affect rooting success. In some cases cold storage is required to improve rooting ability. Fege (1983) recommends storage of *Populus* cuttings at temperatures near freezing for short term handling and at slightly cooler temperatures (-10^0 C) for longer-term storage (five months or more). The cuttings stored at these lower temperatures had higher concentrations of sugars, lower concentrations of starch, and slightly higher total carbohydrates (Fege and Brown 1984). However, storage temperature had little effect on performance of these same clones in subsequent field trials (Fege and Phipps 1984).

Sometimes, chilling can significantly improve rooting success when hardwood cuttings are to be rooted under greenhouse conditions. Sycamore cuttings collected in the fall from mature ortets rooted at 58 percent when treated with auxin and stored at 4^0 C for one month compared with 35 percent rooting for cuttings treated with auxin and immediately set in the greenhouse (Hare and Land 1982). Smith and Wareing (1972b) found that fall-collected *Populus* cuttings given 10 weeks of chilling at 3^0 C produced significantly more roots than unchilled seedlings. The improved rooting may have been a response of increased endogenous auxin levels that were found in chilled compared with unchilled cuttings.

Other storage treatments for hardwoods include warm temperature callusing and bottom heat callusing. These two procedures involve treating dormant fall-collected cuttings with root-promoting chemicals and then either (a) storing them under warm, moist conditions for several weeks followed by planting in the nursery (warm temperature callusing) or (b) storing them under normal winter or cool conditions with the base of the cutting placed in damp packing material over bottom heat (Hartmann et al. 1990). Warm temperature callusing significantly improved rooting success of *Pyrus communis* L. hardwood cuttings. Noncallused controls rooted at 0 and 1 percent in 2 studies, and the best auxin treatments for the callused cuttings in those studies rooted at 45-72 percent (Hartmann et al. 1963).

Rooting of both hardwood and softwood cuttings can be improved by treatment with various chemicals. The most commonly used root-promoting substance is some form of auxin. The literature abounds with reports of successful rooting of species treated with auxins that otherwise would not root or rooted very poorly. Blazich (1988) provides a good summary of the use of auxins to promote rooting and lists four advantages of their use: (a) increased percentage of cuttings that initiate roots, (b) increased rate of root initiation, (c) increased number of roots initiated and quality of roots, and (d) improved uniformity of rooting.

Other plant growth regulators evaluated for their effects on adventitious rooting have provided mixed results. For most of the classes of growth regulators there have been both promotive and inhibitory effects reported. A good summary of research with each class of compounds is provided in the book "Adventitious Root Formation in Cuttings" (Davis et al. 1988).

In addition to treatment with auxins, the leaves on softwood cuttings from species with large leaves are often trimmed to improve water relations. Geary and Harding (1984) compared the rooting of *Eucalyptus camaldulensis* cuttings with trimmed and entire leaves and found that a significantly greater percentage of cuttings with trimmed leaves rooted and flushed. Leakey and Coutts (1989) trimmed the leaves of *Triplochiton scleroxylon* Schumann cuttings to three different sizes (10, 50 or 100 cm^2). After 42 days of propagation, the larger-leafed cuttings had greater dry weights and carbohydrate levels. However, fewer of these large-leafed cuttings rooted, possibly because they had a large leaf area that reduced leaf water potential prior to root emergence. The authors hypothesized that rooting ability is partially determined by a balance between transpiration and photosynthesis.

Environmental Conditions During Rooting

Rooting soil medium, fog or mist systems, temperature, and light in the greenhouse or nursery can affect rooting success. Since the topic is covered

by another paper at this symposium (Ford-Logan 1993), it will not be discussed here.

Planting Methods

Once greenhouse- and nursery-grown cuttings have been rooted, they can be handled similarly to seedlings. Softwood cuttings are typically grown in containers and subsequently field planted when they reach an appropriate size and when soil conditions are suitable (Campinhos and Ikemori 1980, Cunningham 1989). Hardening-off treatments involving reductions in watering and shade are important for these softwood rooted cuttings before planting in the field.

Field-planted unrooted cuttings require good hardwood site preparation prior to planting (Huppuch 1960). In addition, weed control through cultivation or chemicals is essential until crown closure (Kennedy 1975, Huppuch 1960, McKnight 1970). Cuttings have traditionally been planted vertically leaving 2.5-5 cm of the top of the cutting above ground. However, Hansen et al. (1991) have recently reported that planting the cuttings flush with the soil surface gives fewer multiple-stem trees for hybrid poplars, and there are no effects on growth or mortality. There has been some research evaluating the potential of horizontally planted cuttings in furrows. Good success has been obtained with (a) green ash (*Fraxinus pennsylvanica* Marsh.) cuttings derived from seedling sources (Kennedy 1972) and (b) sycamore sprouts (Briscoe 1969). One disadvantage to horizontally planted cuttings is that multiple stemmed trees are usually produced.

In the South, hardwood cutting plantations have been successfully established throughout the dormant season (Briscoe 1963, Briscoe 1973, Nelson and Martindale 1957). Mild winters usually keep the ground from freezing, and root initiation can take place while shoots are still dormant (Nelson and Martindale 1957). In more northern locations where soil freezing and thawing occur, fall plantings can meet with poor success due to frost heaving. Hansen (1986) recommends that spring planting be delayed until soil temperatures are above 10^0 C. When soil temperatures are below this value cuttings will not grow roots, will expend storage reserves, and will experience water stress.

CUTTING MACROPROPAGATION OF SOME HARDWOOD TREE SPECIES

Recommendations for 16 Genera

Summary comments on difficulty of rooting cuttings and on the best methods for maximizing rooting success are presented in Appendix Table 1 for 16 hardwood genera that have been used for wood production in the United States. The difficulty of rooting has been subjectively divided into four classes in that table. Class "A" species are those that are very easily rooted (they usually have preformed root initials on stem cuttings) and can be planted directly in operational field plantations as dormant, unrooted hardwood cuttings. Either juvenile or mature cuttings can usually be used, although cuttings from one-year-old shoots on top-pruned stock plants in stool beds are preferred. Class "B" species are slightly more difficult to root. However, hardwood stem cuttings from juvenile material will root easily without treatment and can be planted as dormant, unrooted cuttings in operational field plantations. Mature material must be either rejuvenated (or invigorated), or the cuttings must be rooted with customized treatments in the nursery or greenhouse, before outplanting. Class "C" species are somewhat difficult to root, but rooting can be accomplished with customized treatments in greenhouses or nurseries (as summarized under "Comments" in Appendix Table 1). Only rooted cuttings of these species could be outplanted operationally. Class "D" species are very difficult to root. In some cases, no reported successes in rooting are available in the literature. Cutting macropropagation on an operational scale is not an option for these species, at least with current technology.

Some Operational Programs

Cutting macropropagation is used operationally for forestry in the United States for *Populus* (*Aigeiros* Duby and *Tacamahaca* Spach sections), *Eucalyptus* (species shown in Appendix Table 1), and *Salix* L. species and hybrids.

Populus deltoides (eastern cottonwood) has long been planted as unrooted hardwood cuttings in the Mississippi River valley. These plantations represent approximately three-fourths of all commercial poplar plantings in the United States (DeBell 1992). One example is the Fitler Managed Forest owned by James River Corporation in western Mississippi, where 45-cm cuttings from one-year-old shoots on stools in a cutting production nursery are collected and planted during the winter. *P. trichocarpa* Torr. and Gray *x P. deltoides* hybrids

are being planted as unrooted 30-40 cm hardwood cuttings in Washington and Oregon. Many of the clones have been developed by the University of Washington/Washington State University Poplar Research Program. The plantations are being grown on a six-year rotation for pulpwood. James River Corporation already has plantings of 3480 ha in the lower Columbia River Valley (some of which are now being harvested), and Boise Cascade Corporation expects to plant 8100 ha in eastern Washington by 1996 (DeBell 1992). Other hybrid poplars (such as *P. deltoides x P. nigra* L., *P.candicans* Ait. *x P. berolinensis* Dippel, and *P. charkowiensis x P. caudina* [= *P. nigra* var *Caudina* Tenore]) (Hansen et al. 1991) are being planted as unrooted hardwood cuttings in many parts of northeastern and north-central United States and southeastern and south-central Canada. An example of the magnitude of these plantings is the Hybrid Poplar Plantation Network, which is a cooperative effort between the U.S. Forest Service, the Department of Energy, Energy Performances Systems Inc., and the Electrical Power Research Institute. Twenty-three plantations in Minnesota, Wisconsin, North Dakota, and South Dakota were being managed in the Network in 1991, and a 20,200 ha plantation to fuel. a 100 megawatt tree-burning power plant was planned for 1992[4]

Rooted cuttings of *Eucalyptus* species are being used by members of the Eucalyptus Improvement Association Inc. in California to establish short-rotation (10-year) plantations for energywood and pulpwood. The primary species for the clonal forestry program are *Eucalyptus camaldulensis*, *E. grandis*, *E. viminalis* Labill, and *E. gunnii* Hook. x *dalrympleana* Maiden (Sachs et al. 1988). There were 4000 growers of Eucalypts in California in 1991 with a total area of approximately 35,600 ha in plantations. In Brazil, Aracruz Florestal company is planting 15 million rooted cuttings per year of *E. grandis* on a six-year rotation for high-quality, bleached sulfate pulp (Ikemori 1984, Zobel 1992, Zobel et al. 1983).

Unrooted hardwood cuttings of a hybrid willow (*Salix matsudana* Koidz. x *alba*) from New Zealand are being produced by Austree, Inc. in Pescadero, California (four nurseries in California and private nurseries in Indiana, Montana, Nebraska, and Saskatchewan). The cuttings are used to rapidly establish windbreak, energywood, and pulpwood plantations and for land reclamation plantings. A single male clone (sterile), trademarked as "Austree", has been planted on a limited scale throughout the United States and Canada. Tree and shrub willows (*Salix alba*, *S. purpurea* L., *S.* x *rubens* Schrank, *S. viminalis* L., *S. amygdaloides* Anderss., *S. bebbiana* Sarg., *S. discolor* Muhl., *S. eriocephala* Michx., *S. exigua* Nutt., *S. lucida* Muhl., *S. pellita* Anderss. ex Schneid., and *S. petiolaris* J.E.Sm.) and their hybrids are being planted as unrooted hardwood cuttings at very close spacings (15-45 cm) by the College of Environmental Science and Forestry at the State University of New York, the Forest Genetics Laboratory at the University of Toronto, and the Ontario Ministry of Natural Resources to test the ultrashort-rotation ("woodgrass") concept of bioenergy plantations[5] . These are small scale, experimental plantings. There are currently no commercial *Salix* bioenergy plantings in the northeastern United States or southern Canada, although Domtar Corporation in Ontario, Canada, established some small experimental plantings of *S. alba* for pulpwood in 1990.

MANAGING WITHIN-CLONE VARIABILITY

All cuttings from the same clone do not perform identically. Within-clone variability can be due to phenotypic (non-persistent developmental and environmental) effects, epigenetic (persistent phase change or gene regulation) effects, somatic genetic changes and chimeras, and systemic or non-systemic pathogenic effects. The objective of vegetative plant propagation is to reproduce, year after year, a specific cultivar that is true-to-type with a minimum of within-clone variability.

Phenotypic (Non-persistent) Variation

Periphysis is the carryover effect of the ortet's (or rootstock's) environment on cutting performance, but without causing a permanent genetic or phase

[4]Reported in Tresearch Newsletter (Volume 2 No.2, Spring 1991) from the Department of Soil Science, University of Wisconsin, 1525 Observatory Drive, Madison, Wisconsin.

[5]Personal letter and progress report provided by Mr. Richard F. Kopp, Senior Research Support Specialist, State University of New York, College of Environmental Science and Forestry, Syracuse, New York. Principal investigators are Drs. Edwin H. White and Lawrence P. Abrahamson.

change (Barnes and Burley 1987, Hartmann et al. 1990). Rootstocks of the same clone that are grown under different environmental and cultural conditions can produce cuttings that perform differently in early growth rates and hardiness. Mixtures of these cuttings can give large within-clone variability. The management questions are (a) what are the best cultural conditions for rootstocks to provide cuttings of uniformly high production potential, and (b) how long do the periphysis effects last? Plantation spacing might be adjusted to reduce the impact of these effects on competition-caused variability. Forestry cutting macropropagation programs should expect that different nurseries, or even in different portions of the same nursery, may have periphysis effects on cutting performance after outplanting. Standardized greenhouse procedures for mass production of rooted cuttings in containers will reduce this effect.

Epigenetic Variation

Epigenetic effects include **cyclophysis** (variation in phase [age] of cuttings from different parts of a tree) and **topophysis** (variation in growth form of cuttings from different parts of a tree) (Barnes and Burley 1987, Hartmann et al. 1990). These persistent, long-term effects are not caused by genetic differences, but can be transferred to later crops of cuttings by serial vegetative propagation. Thus, they are more serious sources of within-clone variation than periphysis effects, and are more difficult to control.

As discussed earlier in this paper, cuttings from different parts of large, mature trees can differ in ontogenetic age and in physiological age. Cuttings from near the base of the stem are more juvenile than those from further up the stem and in the crown (Briscoe 1963, Preece et al. 1991). These are effects of ontogenetic age of the meristem. In addition, there can be differences in physiological age and vigor of cutting sources within the crown. Hare and Land (1982) found that annual growth increment of limbs was positively correlated with rooting success of hardwood cuttings from mature sycamore trees, so that selection of limbs with greater growth increments within the crown gave greater rooting. The correlation was not improved by including tree age (10-60 years old) in the regression equation. As a result of within-tree variation in ontogenetic and physiological ages of cutting sources, the population of ramets produced from the first vegetative cycle of expansion of a mature tree may be highly variable in rooting ability, growth, and morphology. This heterogeneity is transmitted to the cuttings of successive vegetative generations (Franclet 1983). Within-clone selection must be applied (usually for juvenility) to obtain a stool bed population of similar phase for production of uniform cuttings, and this homogeneity in phase must be maintained over successive vegetative cycles by methods of rejuvenation and invigoration.

The other epigenetic effect on variation within clones is topophysis, which occurs extensively in conifers and sometimes in hardwood tree species (Hartmann et al. 1990). Ramets produced from cuttings of upright shoots will grow vertically (**orthotropic**), while cuttings taken from lateral limbs will grow more-or-less horizontally (**plagiotropic**). Careful selection of source of cuttings is required to reduce this type of within-clone variation for those species that exhibit topophysis. Plagiotropic growth in hardwoods has been reported for coffee (*Coffea arabica* L.) (Hartmann et al. 1990), several Eucalyptus species (Durrand-Cresswell et al. 1982), black locust[6] , and (potentially) the tropical hardwood genus *Dipterocarpaceae* Blume. (Barnes and Burley 1987).

Genetic Variation Within Clones

Genetic variation within clones originates from somatic cell mutations that occur during expansion and production of cuttings from the selected clone. These are **bud-sports** or **bud-mutations**, since the mutation occurs in one of the mitotic cells in the apical meristem of a bud. The rate of such mutations is low, but when operational programs produce literally millions of cuttings for propagation the probability for some mutations to occur spontaneously somewhere in the clone is high. Latent mutations, such as those affecting a mature trait, may remain undetected for many years and could be rapidly expanded in a serial propagation system with stool beds.

Because of the arrangement of cells in an apical meristem, mutation of a single cell invariably results in a bud and shoot containing a mixture of mutated and nonmutated tissue (a **chimera**). Most gymnosperms and the roots and young embryos of angiosperms have unstructured apical meristems

[6]Personal communication from Dr. Bruce Bongarten, School of Forest Resources, The University of Georgia, Athens, Georgia.

that result in sectorial chimeras (part of the internal and external tissues are mutated) (Hartmann et al. 1990). However, most angiosperms have structured apical meristems that result in periclinal and mericlinal chimeras (external layer of tissue is mutated and internal core is not, or vice versa). This is important, as the cells used to produce the cutting will determine whether it is a mutant type or nonmutated type. For example, most cuttings involve perpetuation of the apical meristem and would contain the periclinal chimera of mutated epidermis and non-mutated core. If propagation uses adventitious shoots that arise internally, however (such as softwood cuttings from root sprouts), there would be a reversion to the non-mutated genotype of the inner tissue. With sectorial chimeras this would not happen. Thus, in hardwood tree species genetic differences can arise among individual ramets as a result of bud mutations and, subsequently, the source and type of cuttings used.

Variation Caused By Pathogens

Pathogens such as fungi, bacteria, viruses, mycoplasma-like organisms (MLOs), rickettsia-like organisms (RLOs), viroids, nematodes, and insects and mites can infect and be transmitted in cuttings (Hartmann et al. 1990). The pathogens can affect growth, form, or leaf characteristics and thereby contribute to within-clone variability. Some of the pathogens are easily detected from visual examination of cuttings, such as infection of *Populus deltoides* cuttings by wood-boring insects and fungi-caused canker diseases (Morris et al. 1975). Infected cuttings can be discarded during grading at the nursery. However, many pathogens cannot be detected by simple visual examination, and the incidence of these pathogens can rapidly increase in cutting-production systems. Most operational forestry cutting programs rely on what Hartmann et al. (1990) call the "Pedigree Selection" system -- regenerated stool beds are established with cuttings from the previous stool beds, and the only selection is a visual inspection to avoid "off-type" plants or insect/disease-infected cuttings. According to the authors, that system has historically resulted in "... eventual contamination by viruses and other pathogens, which in some instances have threatened the viability of the entire industry."

Management to Control Within-Clone Variability

Operational forestry programs relying on rooted cutting macropropagation should recognize that within-clone variability exists or will occur. They should develop management plans to minimize these effects. Some suggested strategies are given below.

I. Develop stoolbed procedures to produce uniform cuttings, and grade all cuttings at time of collection and packaging.

(a) Use uniform nursery site, uniform spacing among stools, and uniform fertilization and irrigation practices.

(b) For rootstocks in the nursery, plant pathogen-free cuttings that are uniform in size and bud characteristics.

(c) Grade harvested unrooted cuttings by diameter, length, bud characteristics, and absence of visible infection at time of packaging, and discard those that don't meet standards.

(d) Before outplanting rooted cuttings, grade by sprout size, number and distribution of roots (if bare-root cuttings), and absence of visible infection. Discard those that don't meet standards.

II. Develop a "foundation stock" of a limited number of pathogen-free, genetically true-to-type ramets of each clone.

(a) Molecular markers might be used to determine if each ramet of a clone is genetically identical and if each cell layer in the apical bud has the same genotype.

(b) Shoot-apex culture or heat treatments (Hartmann et al. 1990) could be used to obtain pathogen-free material from infected material.

(c) For species with recurrent apomixis (such as apple), apomictic seeds having the same genotype as the mother tree can be used to produce pathogen-free, juvenile individuals.

III. Maintain the foundation stock in one or more locations under protected conditions to prevent infection by pathogens. The foundation stock should be located at least 1/2 mile from the production nursery and plantations, in order to separate the plants from infective agents (particularly for insect-, nematode-, or pollen-vectored diseases and viruses).

IV. Change field position within the nursery each time stool beds are regenerated. The old stoolbed site should be cleared of all rootstock, fumigated,

and used for other crops for several years before being used again as a stoolbed.

V. If a decline in growth or an increase in within-clone variability of cuttings occurs after several cycles of stoolbed regeneration with cuttings from the previous stool beds, start a new stool bed with cuttings from the foundation stock. Use good sanitary procedures (such as repeated alcohol dips of saws and use of sterilized rooting containers and rooting media) in collecting and expanding cuttings from the foundation stock.

VI. Maintain juvenility in the foundation stock by cutting back tops to produce stump sprouts each year, and re-establish rootstocks every 2-3 years from the stump sprouts or from root suckers. Use good sanitation procedures to prevent spread of pathogens.

VII. Continually monitor foundation stock for infection or bud-mutation.

(a) Remove any "off-type" plants observed in visual inspections, check for non-visible mutations with molecular-marker analyses, and develop culture-indexing or virus-indexing methods (Hartmann et al. 1990) for detecting the presence of latent pathogens.

(b) If any pathogen contamination is detected, then develop a new, pathogen-free foundation stock by shoot-apex culture and/or heat treatments (Hartmann et al. 1990).

SUMMARY

Rooted cuttings can be obtained from most hardwood tree species, but with widely varying degrees of rooting success. Literature cited and data presented in this paper indicate that chances of success are increased by the following:

(a) use juvenile shoot or root cuttings, either from young plants, from sprouts arising near the base of older trees, or from rejuvenated (or invigorated) tissue;

(b) produce shoot or root cuttings in stool beds where high C/N ratios in stock plants and reduction in stock plant irradiance may be accomplished;

(c) select individual clones for high rooting ability;

(d) collect the shoot or root cuttings in the most appropriate season for that particular species (hardwood versus softwood for shoot cuttings);

(e) select hardwood shoot cuttings from the base of sprouts, while softwood cuttings should come from shoot tips;

(f) retain trimmed leaves on softwood shoot cuttings, and for hardwood cuttings use large cuttings (13-25 mm diameter) that have multiple (>2) nodes per cutting (with one near the base and one near the top);

(g) for some species, provide a cold storage or warm temperature/bottom heat callusing interval (with or without an auxin chemical dip, depending on species) between collection and planting of hardwood cuttings; and

(h) plant rooted or unrooted cuttings on intensively prepared sites in the field after the danger of frost heaving is past, followed by thorough weed control until crown closure.

Some species in 16 hardwood genera have been classified into four classes based on the relative ease of obtaining rooting success. Class "A" species are those that root very easily (usually have preformed root initials on stem cuttings) and can be planted directly in the field as unrooted hardwood cuttings. The genera *Platanus* L., *Populus*, and *Salix* contain these species. Not surprisingly, species and hybrids of *Populus* and *Salix* are involved in operational cutting macropropagation programs in the United States. At the other end of the range in ease of rooting are the class "D" species. These species are very difficult to root, and cutting macropropagation on an operational scale is not an option with the current level of technology. The genera *Carya* Nutt., *Fraxinus* L., and *Juglans* L. contain many of these species.

Managing within-clone variability will be a concern in cutting macropropagation programs, once the methods for obtaining and maintaining high rooting success have been developed. The objective of these programs is to consistently reproduce a specific cultivar that is genetically true-to-type and has a minimum of within-clone variability. Sources of variability include (a) phenotypic (non-persistent,

non-genetic) variation caused by environmental differences among different rootstocks of the same clone, (b) epigenetic (persistent, non-genetic) variation caused by differences in degree of juvenility or growth habit of cuttings from different parts of a tree, (c) genetic differences arising from mutations within a clone, and (d) differences caused by pathogen infections of some of the cuttings or rootstocks from a clone. A management strategy to control within-clone variability is suggested that would include (a) development of stoolbed procedures to provide uniform environmental conditions for all rootstocks, (b) grading all cuttings for uniformity at time of collection and packaging, (c) development and maintenance of a pathogen-free, genetically true-to-type, juvenile foundation stock of all clones at a different location from the production stoolbed nursery, (d) rotating field positions in the nursery each time the stool beds are regenerated, and (e) starting a new production nursery with cuttings from the foundation stock, using good sanitary procedures to prevent pathogen infection, if the old production nursery begins to decline in growth or increase in within-clone variability.

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Appendix Table 1.--Summary comments and references for rooted cutting macropropagation of 16 hardwood genera.

Genus	Species	Difficulty of Rooting[1]	Comments	Ref. (by no.)
			Type of cutting/when to collect/type & conc. of hormone	
Acer (maples)	*negundo* L. *rubrum* L. *saccharinum* L. *saccharum* Marsh.	B B B C	Rooting of juvenile and mature material is successful for *negundo*, *rubrum*, and *saccharinum*, but *saccharum* is more difficult and must use juvenile cuttings. For *rubrum* and *saccharinum*, juvenile cuttings may not need growth hormones, but treat single-node cuttings of mature trees with either 8000ppm NAA quick-dip, or with IBA or K-IBA at 3000 to 6000ppm quick-dip, or treat 23 cm cuttings with 2-4 nodes with 1000ppm IBA and 500ppm NAA solution, and place under mist over bottom heat. Timing is critical, for cuttings collected in August or later have poorer rooting. Semihardwood cuttings collected in September and treated with 8000ppm IBA-talc rooted readily for *negundo*. Softwood juvenile cuttings collected in June gave some rooting success for *saccharum*, wounding did not help, and hormones did not improve success in some reports and did (0%-4% quick-dip IBA solution) in others. Much tree-to-tree variation in rooting ability of *rubrum*.	1,17,18, 41,62,70
Alnus (alders)	*incana* Moench. *glutinosa* Gaertn.	C C	Use 8000 ppm IBA-talc for stem cuttings (*incana*) or June-July softwood cuttings (*glutinosa*); untreated cuttings don't root.	17,18,41
Betula (birches)	*nigra* L. *papyrifera* March.	C C	Timing is very important, and any period of dryness on the leaves' surfaces insures failure; use 1000ppm IBA in 50% alcohol (or K-IBA) on June-July softwood cuttings (*nigra*), and either 2000ppm IBA-alcohol quick-dip or 8000ppm IBA-talc on wounded semihardwood cuttings taken in August-September (*papyrifera*). One study of *papyrifera* indicated low rooting success for softwood cuttings (1500ppm K-IBA quick-dip) from grafted scionwood of mature trees, that hedging and defoliation of grafts were not effective, but that serial propagation (using previously rooted cuttings as stock plants) more than doubled rooting success (from 20 to 44%).	17,18,41, 71
Carya (pecans, hickories)	*illinoensis* K.Koch (many others)	D D	No reports of successfully rooted cuttings of hickories; minimal success for pecan -- best for juvenile wood and root cuttings, using 1% IBA-talc and/or 1000ppm IBA-solution.	17,18,41

[1] A = Hardwood cuttings from juvenile and mature material will root without rooting hormones and can be planted directly in the field;
B = Cuttings from juvenile material may root readily without hormones, but cuttings from mature material require specific treatments/conditions to get high (>50% rooting);
C = Cuttings from juvenile material require specific treatments/conditions to give high (>50%) rooting success, but cuttings from mature material provide only low to moderate (10%-50%) rooting success even under the best treatments/conditions; and
D = Cuttings from juvenile and mature material are difficult to root (<10%) under any treatments/conditions.

Eucalyptus (eucalypts)	*grandis* Hill ex Maiden. *camaldulensis* Dehn. *tereticornis* Sm. *gunnii* Hook. *cladocalyx* F.Muell. *deglupta* Sm. *robusta* Sm. (many others)	C C C C C B B	Eucalypts are considered difficult to start from cuttings as a genus; there is large variation in rooting ability among species and within species; 78 species reported to form roots on stem cuttings taken from seedlings or basal epicormic shoots; in general, it is relatively easy to propagate by cuttings provided leafy (firm softwood) cuttings are taken from very young seedlings or epicormic shoots at the base of the tree (sometimes produced by partial girdling); timing is important -- best in spring or fall when growth rate high (California) or when cuttings not too woody or too succulent during growing season (Brazil); 4-node softwood cuttings with trimmed leaves best; dip in 6000 ppm IBA-talc or quick-dip in 3000-8000ppm K-IBA solution and stick in vermiculite:perlite (not peat) under mist (must keep leaves moist); may use bottom heat (California); rooting in 2-5 wks. and planted to field in 10-12 wks.; problems are plagiotrophic growth (*cladocalyx*) and unbalanced distribution of roots (wind throw).	1,11,19, 27,28,41, 42,44,53, 55,61,64, 74,75
Fraxinus (ashes).	*americana* L. *pennsylvanica* Marsh.	D C	Cuttings of *americana* have been nearly impossible to root, even from young trees; horizontal planting of seedling cuttings has been successful for *pennsylvanica*.	18,46
Juglans (walnuts)	*nigra* L. *regia* L.	D D	Cuttings are very difficult to root; some success for cuttings from seedling plants, but poor from older trees (unless rejuvenated by serial grafting on young seedlings [*nigra* x *regia*]); etiolated shoots from juvenile plants girdled and 1% IBA-lanolin put above girdle in April-June, covered in foil, and subsequently severed from plant to give some rooting (*nigra*); softwood cuttings rooted for *regia*, but rooted cuttings can't be disturbed and survival is poor.	18,22,27, 41
Liquidambar (sweetgums)	*formosana* Hance *styraciflua* L.	C C	Softwood cuttings from adventitious shoots on root segments can be rooted; softwood cuttings collected from shoot tips on crowns and stump sprouts of mature trees in early June, treated with 4000ppm IBA-solution or 8000ppm IBA-talc, and placed in peat:perlite under mist root well; overwintering may be a problem. Hardwood cuttings will not root unless girdled, treated at the girdle with IBA + PPZ [1-phenyl-3-methyl-5-pyrazolone] + sucrose + captan, and subsequently severed at the girdle before planting; etiolated shoots don't improve rooting.	5,13,17, 18,20,36, 41,49
Liriodendron (yellow poplar)	*tulipfera* L.	C	Considered difficult to root, particularly from mature plants (juvenility is important if species is to be rooted in high percentages). Leafy stem cuttings (tree age unknown) collected in July with the basal cut 1 cm below a node rooted 52%. Root cuttings and cuttings from stump sprouts have been rooted. Partial girdling tree and using basal sprouts has increased rooting, but etiolated shoots don't root.	17,18,41, 49,54

Platanus (sycamores)	x *acerifolia* Miller ex Munchh.	A	Cuttings of x *acerifolia* provide high rooting success, even from trees as old as 45 yrs (95% rooting with no hormone trt) and from unrooted hardwood cuttings planted directly in the field (70% rooting and survival); NAA is valueless, and IBA is no better than the untreated control; leafy June softwood cuttings collected after the shoots have begun to mature can be rooted, and success is highest with cuttings taken from young vigorous stock plants cut back annually during the dormant season ("invigoration"); hardwood cuttings should be taken after leaf fall in the autumn from the current season's growth and be 15-23 cm long with 4 buds (top cut just above a bud and lower cut just below a bud). Cuttings of mature *occidentalis* are more difficult to root than x *acerifolia*, but juvenile cuttings (1-yr-old seedlings and coppice stump sprouts on young stock plants) root easily and can be planted directly in the field as unrooted hardwood cuttings; 58% of hardwood cuttings collected from mature trees were rooted when treated with a quick-dip of 0.5% IBA + 0.5% PPZ [1-phenyl-3-methyl-5-pyrazolone] in 50% ethanol, then dipped in 20% powdered sucrose and 5% captan in talc, and put in cold storage for one month before planting; rooting success for hardwood cuttings from 1-yr-old sprouts on mature, pruned trees was highest for 25-cm cuttings having a basal diameter of 15-20 mm, 2-3 nodes, and the lowest node within 2 cm above the basal cut. Reduced N fertilization of stock plants and growing them in full sun may increase rooting of cuttings; horizontal planting of cuttings from sprouts has provided successful rooting.	7,8,9,12, 17,18,27, 35,38,41, 43,50,51, 60
	occidentalis L.	B		
Populus (poplars, cottonwoods, and aspens)	*Aigeiros* Duby:		Hardwood cuttings of most poplars (*Aigeiros*, *Tacamahaca*, and *Leucoides* sections, and the species *alba*) root readily, but not the gray poplars and aspens (see below); cuttings are usually 25-45 cm pieces (6-19 mm diameter) of dormant 1-yr-old shoots collected from coppiced stock plants in stool beds (not collected before October in Minnesota) that may be stored in a cooler (-10 C) for up to six months and are then planted in the field as unrooted cuttings during the early spring (soil temperature >10 C) in the northern U.S. (all winter in the South, without cold storage); cuttings from the base of stoolbed sprouts root and grow better than cuttings from distal portions, because they have more preformed root initials; roots form from these root initials to give 90% to 100% rooting success; however, greater uniformity in growth and fewer multiple stems are obtained when cuttings have healthy axillary buds (not from branched portion of stem and not from stem tips) and are planted so that the top of the cutting is flush with the soil surface; weed control is essential after planting. The gray poplars and aspens (x *canescens*, *grandidentata*, and *tremuloides*) can be propagated from root cuttings and from softwood cuttings from root suckers (5-10 cm root pieces collected and placed in moist peat moss in February, and soft shoots that develop on these root pieces by late March can be rooted under mist with bottom heat and IBA-talc). Aspens and *Leucoides* poplars root least readily from softwood cuttings, while *Aigeiros* and *Tacamahaca* poplars are easiest to root.	4,7,15, 16,17,18, 23,24,25, 27,31,32, 33,41,47, 56,63,66, 68,69,72, 73,76
	deltoides Bartr. ex Marsh.	A		
	nigra L.	A		
	Tacamahaca Spach:			
	balsamifera L.	A		
	trichocarpa Torr. and Gray	A		
	Leucoides Spach:			
	heterophylla L.	B		
	Leuce Duby:			
	alba L.	A		
	x *canescens* (Ait.) Sm.	C		
	grandidentata Michx.	C		
	tremuloides Michx.	C		
Prunus (cherries)	*serotina* Ehrh.	C	Softwood cuttings collected in spring and early summer from (a) root suckers on root cuttings, (b) seedlings, and (c) mature trees (which were grafted in pots and pruned to promote shoot formation) have been rooted with 8000ppm IBA-talc under mist in sand:peat. Wide tree-to-tree variation was observed in rooting success (0-100%).	18,21,27
	avium L.	C		

Quercus (oaks)	*laurifolia* Michx. *lyrata* Walt. *nigra* L. *palustris* Muenchh. *phellos* L. *robur* L. *shumardii* Buckl. *virginiana* Mill. (other oaks)	C C C C C C C C D?	Attempts to propagate oaks by cuttings have usually been failures,but the species listed here (except *nigra*) have been successfully rooted as juvenile leafy, semihardwood cuttings. Juvenility is most important. Semihardwood cuttings collected in July (firm cuttings after first growth flush hardens), quick-dipped in 10000-20000 K-IBA solution, and placed in 2 perlite:1 peat under mist and 50% shade gave 60-80% rooting in 7-10 wks. Girdling stock-plant shoots and treating the girdle with IBA + PPZ + sucrose + captan before severing the shoot increased rooting (76%) of 19 to 57-yr-old *nigra* trees. Basal sprouts from partially girdled *robur* have been rooted.	17,18,37, 39,41
Robinia (black locust)	*pseudoacacia* L.	B	Root cuttings of 7-13 mm diameter and 10 cm length have given greater than 90% rooting success in Hungary when dug in the early spring and planted directly into nursery rows with the distal end down. Softwood cuttings from coppice sprouts can be rooted under mist with near 100% success. Rooting media containing high levels of peat should be avoided, as cuttings are sensitive to excessive moisture. Hormones are not required, but quick-dip in 10000-15000 IBA in ETOH can improve rooting slightly. Plagiotropism is a problem, unless softwood cuttings are taken from coppice sprouts or root suckers.	17,18,41, 48 [2]
Salix (willows)	*nigra* Marsh. *alba* L. (many others)	A A	Willows root readily by either hardwood or softwood stem cuttings (have preformed root initials) or by root cuttings. Hardwood stem cuttings can be collected anytime after leaf fall, but fall-collected cuttings may root more poorly than spring-collected cuttings and require chilling or removal of buds to improve rooting success. Spring cuttings (and chilled fall cuttings) can be stuck directly in the field. Rooting success is 90-100%, even for very large cuttings (survival and growth of *alba* increases with increasing diameter up to 19 mm, and not affected by diameter above that size). Rooting hormones don't help. Softwood cuttings can be rooted in high percentages in peat:perlite under mist. A "willow rooting cofactor" is present in willow stems and has sometimes been used to promote rooting of hard-to-root plants.	7,10,17, 18,41,45, 76
Ulmus (elms)	*alata* Michx. *americana* L.	C C	Elms can be propagated from root cuttings, softwood cuttings from root cutting sprouts, and softwood leafy stem cuttings. Root cuttings are collected in December-January, cut into 5-8 cm lengths, dusted with captan, and placed in rooting medium over 20°C bottom heat. When root sprouts become woody, they are treated with 8000ppm IBA-talc and put under mist. Semi-lignified softwood leafy stem cuttings that were collected in early June, cut into 5-10 cm lengths with 1+ leaves, treated with 8000ppm IBA-talc, and stuck in peat:perlite under mist rooted 94%. Moisture during the period immediately after insertion in the rooting medium is critical. Also, the juvenility factor is very important in elm rooting success, as stump sprouts from 30 cm above ground gave 83% rooting, stump sprouts from 1.8-2.3 meters above ground gave 64%, and cuttings from mature trees gave 38%. Roots on rooted cuttings are very brittle and can be easily damaged, so rooting in individual pots is recommended.	17,18,29, 41

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THE ROOTING ENVIRONMENT FOR CUTTINGS FROM FOREST TREES[1]

Jane L. Ford-Logan[2]

Abstract.--This paper reviews the requirements of the rooting environment as they affect the induction of adventitious roots on cuttings, as reported from studies on various forest tree species. Evidence for the involvement of growth regulators in root induction is discussed. The environmental conditions which promote rooting include a porous rooting medium, intermittent mist, bottom heat, extended photoperiods, and relatively low light. High humidity is a critical factor in maintaining tissue turgidity until new roots have developed. Both total irradiance and light quality are important components of the stock plant environment, and these factors interact with nutrient availability to influence subsequent rates of net photosynthesis and rooting of cuttings. Rooting medium combinations of sphagnum peat, perlite, and sand appear to give fairly high rooting success for cuttings of forest tree species. Optimal rooting environments enhance the efficiency of rooted cutting production systems, which subsequently encourages the use of vegetative propagules in forest tree improvement programs.

INTRODUCTION

Clonal reforestation requires the development of economically efficient production systems for vegetative propagules. The potential benefits of clonal propagation of forest trees, particularly conifers, to forestry have been discussed by many authors (Rediske 1978, Libby and Rauter 1984, Boulay 1987, Greenwood et al. 1991). The realization of these benefits is hampered by the lack of suitable technology for vegetative propagation of many coniferous species. Progress is continuing in the search for practical, efficient methods to clonally propagate by means of rooted stem cuttings (Rauter 1983, Foster and Shaw 1987, Williams 1987), needle fascicles (Rudolph and Nienstaedt 1964, Cohen 1975, Struve and Blazich 1984, Koh et al. 1990), and by cell, tissue, and organ culture (Mott 1981, David 1982, Bonga 1983, Amerson et al. 1984, Amerson et al. 1988). Rooting cuttings and micropropagation are the two most important methods for cloning that can be employed in the operational production of forest tree propagules.

Propagation of most conifers and hardwoods by rooted cuttings is difficult and variable in success. The ability of plants to form adventitious roots is controlled by a complex array of interacting factors, including nutrition, environment, genetics and other factors relative to the plants, and numerous endogenous and exogenous compounds (Hartmann et al. 1990). This array of factors becomes ineffective as a trigger for adventitious root formation if any one of them is limiting.

One of the most fundamentally important and least experimentally studied aspects of adventitious

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root formation is the rooting environment. Very little literature exists on the environmental conditions needed to induce roots on cuttings of conifers and hardwoods. What is reported on this aspect of vegetative propagation generally concerns herbaceous or softwood cuttings from horticultural species.

The purpose of this paper is to review what is presently known about the requirements of the rooting environment for the propagation of forest trees. This paper is written to document specific points, rather than generalities from past research, and to further improve the understanding of the rooting environment.

Background Studies

Comprehensive studies of environmental factors which promote rooting of cuttings of forest tree species have included *Pinus spp.* (Hare 1974), Douglas-fir (*Pseudotsuga menziesii* [Mirb.] Franco) (Brix and Barker 1973), western hemlock (*Tsuga heterophylla* [Raf.] Sarg.) (Brix and Barker 1975), loblolly (*Pinus taeda* L.) and slash pine (*Pinus elliottii* Engelm.) (van Buijtenen et al. 1975), and southern pines (Marino 1982).

WATER RELATIONS

Water Quality

Water quality is an important factor in rooting cuttings (Hartmann et al. 1990). For good results the available water should not contain total soluble salts in excess of 1400 ppm -- ocean water averages about 35,000 ppm. The salts are combinations of such cations as sodium, calcium, and magnesium with such anions as sulfate, chloride, and bicarbonate. Water containing a high proportion of sodium to calcium and magnesium can adversely affect the physical properties and water-absorption rates of rooting media and should not be used for irrigation purposes.

Problems can arise in maintaining the proper calcium/magnesium levels, both being important elements for plant growth, when soilless media are used. Purified water may not contain adequate amounts of these elements, requiring supplementary fertilizers be added when calcium is 25 ppm or less and the magnesium level is 15 ppm or less.

Hard water containing relatively high amounts of calcium and magnesium (as bicarbonates and sulfates) can be a problem in mist-propagating units. Hard water softened by replacement of the calcium and magnesium in the water by sodium ions is toxic to plant tissue and should never be used for irrigation or misting.

Better, but more expensive methods of improving water quality include deionization or reverse osmosis. Deionization removes calcium, magnesium, and sodium by substituting hydrogen ions for these elements. Reverse osmosis changes a more concentrated solution to a less concentrated solution eliminating unwanted salts from an otherwise good water source (Barnstead 1971).

Humidity

The environmental requirements for adventitious root initiation in stem cuttings are those that minimize physiological stress in the cutting. Providing a high relative humidity to reduce transpiration losses is one means of reducing stress. By doing so, the vapor pressure of the atmosphere surrounding the cutting is maintained close to that in the intercellular spaces of its leaves. Relative humidity is defined as the ratio between the actual weight of moisture and the total amount of water that can be held by a unit volume of air at a specific temperature and pressure expressed as a percentage (Mastalerz 1977).

There are numerous propagation systems used in commercial horticulture to maintain high humidity, which are usually based either on spraying mist, fogging, or enclosing the cuttings in polyethylene. The advantages of polyethylene systems have been known for many years (Loach 1977). Low-pressure misting systems for maintaining a film of moisture on cutting surfaces without saturating the rooting medium have been greatly enhanced and simplified.

Mist vs. No-mist Propagation

There are many cases where mist has been found to be beneficial or essential for rooting forest tree species. Examples include loblolly pine and slash pine (van Buijtenen et al. 1975), and eucalyptus (Reuveni et al. 1990). In contrast to the norm, the use of intermittent mist or fog generated high humidity growth chambers, there are cases where these can be disasterous to a rooted cutting production operation.

Leakey et al. (1990) experimented with a wide range of timber and multi-purpose tree species from both tropical moist forests and semi-arid areas. They compared rooting under mist with rooting in an improved, low-technology, no-mist propagator, and demonstrated the advantages of conditions without mist. A third treatment in which a mist propagator was enclosed in polyethylene, resulted in even less rooting and higher mortalities due to rotting. No-mist propagators used in Kenya to produce clonal material of *Prosopis juliflora* have achieved success rates of greater than 75%. Similarly, cuttings of *Acacia tortilis* and *Terminalia spinosa* were rooted more easily in the high humidity conditions of a no-mist propagator. When the rooting of cuttings of *Ricinodendron heudelotii* was tested both under mist and in no-mist propagators with 'Seradix 2' commercial rooting powder, rooting by day 21 was best without mist (75% vs 50% under mist). In a parallel study on rooting media, a comparison between juvenile *Gmelina arborea* cuttings set in fine sand under mist and the no-mist propagator, showed better rooting in the no-mist propagator.

There are certain positives ascribed to the use of "low-tech," no-mist propagators (Leakey et al. 1990). These high humidity polyethylene propagators are inexpensive to construct, are very effective and have no essential requirements for either piped water or electricity. The advantage seems to be particularly great for the dry-zone species which can be very susceptible to rotting under mist. By being enclosed and continuously moist, the cuttings are not subjected to the extremes of saturation and water stress that can occur when misting frequency is not timed to match changes in the weather.

Brix and Barker (1975) conducted humidity control studies comparing rooting in western hemlock using three humidity control systems: an intermittent misting system with a Geiger Mist-A-Matic control (HC1), flats with cuttings enclosed in clear plastic sheeting (4 mil) and placed under intermittent mist (HC2), and a plastic enclosed box with no mist and shaded from direct sunlight (HC3). Neither heated air or bottom heat was provided to the cuttings, other than that required to keep the greenhouse frost-free. They evaluated six temperature conditions using the three humidity control systems (Table 1). Rooting averaged 33% for Regime 1, 42% for Regime 2, and 45% for Regime 3. Since there were no statistically significant differences among regimes, Regime 3 using plastic covered boxes with no mist was eventually adopted as the standard system.

Table 1.--Temperature conditions and humidity controls for cuttings of western hemlock (adapted from Brix and Barker 1975)

Regime No.	Temperature Condition	Humidity Control
1	unheated greenhouse, unheated soil[1]	intermittent mist (HC1)
2	unheated greenhouse, unheated soil[1]	flats covered under mist (HC2)
3	unheated greenhouse or outside box, unheated soil[1]	box plastic covered, no mist (HC3)
4	warm greenhouse (20°C), warm soil (20°C)	intermittent mist (HC1)
5	unheated greenhouse or outside box, warm soil (20°C)	box plastic covered, no mist (HC3)
6	unheated greenhouse, warm soil (20°C)	intermittent mist (HC1)

[1]Thermostat set at 2°C to keep soil frost-free.

Quantity of mist fall is an important influence on the rooting of loblolly pine cuttings. Greenwood et al. (1980) found that too much mist inhibited rooting of loblolly pine cuttings. Sixty percent rooting was achieved in 4-year old loblolly pine cuttings with a misting regime of 0.05 to 0.1 mm/h. Variation in mist fall accounted for 75% of the variation in rooting observed, with 64% of cuttings rooted in a portion of the bench receiving 0.05 mm/h of mist and 16% in an area receiving 0.77 mm/h, demonstrating that low quantities of mist resulted in the best rooting.

LIGHT

Light is necessary for photosynthesis, and for this reason it is usually also necessary for a good rooting response. Light requirements with respect to rooting cuttings of forest trees are difficult to ascertain due to little work being carried out with these species. In order to understand its effect on

rooting, light must be interpreted with respect to photoperiod (daylength/duration), intensity (irradiance/photon flux), and quality (wavelength).

Photoperiod

The German plant physiologist, Sachs (1882) postulated the existence of a specific root-forming substance manufactured in the leaves, which moves downward to the base of the stem, where it promotes root formation. Later, it was shown (van der Lek 1925) that vigorous bud growth promotes the development of roots just below the buds in cuttings of some plants. Bouillenne and Went (1933) and Went (1934) found substances in cotyledons, leaves, and buds which stimulated rooting of cuttings and called this material "rhizocaline". Removal of the buds from cuttings in certain plants or leafless cuttings almost completely stopped root formation (van der Lek 1925, Leakey et al. 1982), especially in species lacking preformed root initials. This response was shown in hardwood cuttings (Fadl and Hartmann 1967). If hardwood cuttings are taken in midwinter when the buds are in the rest period, they have no stimulating effect on rooting, but if the cuttings are made in early fall or in the spring, when the buds are active, they show a strong root-promoting effect.

Photoperiod influences cambial and bud activity as well as rooting of cuttings of woody plants (Nitsch 1957b). Wareing and Smith (1963) concluded that photoperiod affects rooting either by influencing the activity of the shoot apex or the hormone production in leaves. Leaves and buds are known to be powerful auxin producers, and the effects were observed directly below them, showing that polar transport was involved. The fact that auxin is involved in the process of adventitious root initiation is well established (Blakesley et al. 1991).

Baker and Link (1963) studied the effect of natural daylength, 18-hr, and 24-hr photoperiods on the rooting of cuttings of 26 woody species, and observed an earlier breaking of rest with extended photoperiods in the propagating bench. They found photoperiod had little influence on rooting; however, when dormancy was a factor, extended photoperiods exerted some root-promoting effect.

Similar results were seen in a study conducted by Miller et al. (1982) examining rooting capacity and visible terminal bud activity of Fraser fir (*Abies fraseri* [Pursh] Poir.) stem cuttings as influenced by duration of postseverance chilling and photoperiod during rooting. Cuttings received SD (short-day) or LD (long-day) treatment during a 10-week propagation period. There was no significant relationship between photoperiod during rooting and the number and length of roots per rooted cutting. Rooting was primarily contingent upon IBA (indolebutyric acid) treatment and chilling, although long-days had a strong promotive effect when cuttings were chilled less than 6-weeks. This study, and the one by Baker and Link (1963) showed that long-days may compensate for a shortened chilling or rest period in the rooting of cuttings of some woody species.

Snyder (1955), working with cuttings of Japanese yew (*Taxus cuspidata*) taken in December, found that an 18-hr photoperiod stimulated shoot growth of cuttings while in the bench, whereas no bud activity occurred with 8-hr photoperiods. He found no difference in rooting between the two photoperiods. Waxman (1957) reported summer cuttings of flowering dogwood (*Cornus florida*) produced twice as many roots in an 18-hr photoperiod as in a 9-hr photoperiod, and 1 1/2 times as many roots as cuttings under normal daylengths.

Lanphear and Meahl (1961) observed that juniper (*Juniperus horizontalis* "Plumosa") cuttings rooted significantly better under 24- (77.8%) and 18-hr (79.2%) photoperiods than under normal daylengths (40.3%) when rooted during the autumn. They reported that cuttings under these three photoperiods showed 68.8, 31.2, and 0.7% bud break, respectively. According to Nitsch (1957a), cutting growth of staghorn sumac (*Rhus typhina*) and sycamore (*Platanus occidentalis* L.) was stopped completely under SD (10-hr), whereas under LD (14-hr or longer) growth was vigorous. Wareing and Roberts (1956), working with black locust (*Robinia pseudoacacia* L.), concluded that long days maintain cambial activity, whereas short days tended to decrease the activity.

A study by Bhella and Roberts (1974) determined the effects of photoperiod in combination with rooting temperature on bud and cambial activity and subsequent rooting in stem cuttings periodically sampled from field-grown Douglas-fir. An 18-hr photoperiod significantly increased cambial activity, rooting (percentage and quality), bud respiration, and also hastened bud break of stem cuttings as compared with cuttings propagated under 9-hr photoperiod. They concluded that cambial stimulus from leaves under LD may be due to high levels of auxin resulting from such photoperiods, and

conversely, the cessation of cambial activity under SD may result from insufficient auxin production under such conditions. Rooting response was modified by sampling date and temperature of the rooting medium with rooting being the least from August-November (0-4%) and greatest in December and January (15-26%). Their results with Douglas-fir are in agreement with those of Lanphear and Meahl (1961) for juniper, and with Wareing and Smith (1963) for poplar (*Populus robusta*). Van Buijtenen et al. (1975) also reported significantly better rooting of loblolly and slash pine cuttings under long rather than short photoperiods.

Haugh (1978) elucidated that the LD (18-hr) enhancement of rooting reported by Bhella and Roberts (1974) was not a photoperiodic response, but a beneficial effect of greater accumulations of photosynthate, the results of which were higher levels of starch and enhanced root regeneration. He attributed the absence of rooting in September, even in the presence of increased starch content, to the lack of root initiation.

Root initiation can proceed if a correct balance of nutrients (carbohydrates - particularly starch and proteins) and rooting co-factors are available (Stoltz and Hess 1966a, 1966b, Hess 1969, Jain and Nanda 1972). An increase in root initiation with LD was correlated with increased carbohydrates (Hess 1969) and a higher auxin content (Wareing and Smith 1963, Heide 1964).

Intensity

Light may also have negative effects on rooting as shown in earlier investigations (Kawase 1965, Hansen and Eriksen 1974). With leafy aspen (*Populus spp.*) cuttings, a low (8 W m^{-2}) light intensity was found to give the best rooting (Eliasson and Brunes 1976). High (40 W m^{-2}) light intensity decreased rooting when applied to the stock plants and to the cuttings during the rooting period.

Eliasson et al. (1977) illustrated this same effect with experiments using cuttings from 6-week old seedlings of Scots pine (*Pinus sylvestris* L.). Seedlings were grown and the cuttings rooted at the same two light intensities (8 and 40 W m^{-2}). The light treatment during seedling growth had the strongest influence on the subsequent rooting, rather than light treatment during the rooting period. Similarly, cuttings from seedlings grown at low light intensity rooted faster and formed more roots than cuttings from the seedlings grown at high light intensity (Hansen et al. 1978). Photosynthesis decreased in all treatments during the rooting period. There was an increase in dry weight and starch content in cuttings rooted at the low light intensity. This showed that the low light intensity used in this experiment was more than sufficient for production of assimilate for growth and rooting of pine cuttings.

Eliasson et al. (1977) found higher levels of the auxin IAA (indole-3-acetic acid) in cuttings grown at low light intensities than in cuttings grown at high light intensity. The cuttings also showed a positive rooting response to an external supply of the auxin IBA. These results indicate that light influences the hormonal relationships of the cuttings. At least some of the inhibitory effect of high light intensity may be due to a changed hormonal balance.

There is no generally accepted hypothesis for the adverse effect of high light intensities on rooting. This inhibitory effect of light has been found to be strong in some other species, including Norway spruce (*Picea abies* [L.] Karst.), especially if the stem base of the cutting is illuminated during the rooting period (Eliasson et al. 1977). Light influences many biochemical, physiological, and anatomical properties of plants. The best compromise may be to optimize light intensity for photosynthesis and growth, but endure the small negative effects on rooting.

Quality

Stoutemyer and Close (1946) reported radiation in the orange-red region of the spectrum seems to favor rooting of cuttings more than that in the blue region, but there are conflicting reports on the influence of light quality on both stock plants and on the rooting of cuttings (Hartmann et al. 1990). In contrast, Shapiro (1958) reported red light (above 680 nm) to be more inhibitory to rooting than blue, green, or far-red light on stem cuttings of Lombardy poplar (*Populus nigra* "italica").

TEMPERATURE

Air Temperature

Air temperature in combination with bottom heat is important for rooting. Ambient air temperatures can influence the length of time required for rooting. Ideal temperatures appear to be in the range of 20°C

to 22°C for many forest tree species (Rauter 1983) even though Rauter (1971) noted no detrimental effects on rooting spruce (*Picea spp.*) cuttings exposed to temperatures ranging from 10°C to 32°C. Low temperatures increase the length of time required to root cuttings. Generally cuttings root well in cool, moist air surrounding the tops, and warm conditions around the base. This temperature gradient allows greater activity at the base, while minimizing respiration and moisture stress at the top of the cutting.

Brix and Barker's (1975) rooting studies of western hemlock cuttings evaluated the effect of temperature of the environment during rooting. They evaluated six temperature conditions using three humidity control systems (Table 1). The most consistently good results were obtained when neither the air nor the soil was heated. Regime 4 (warm air and warm soil combination) was generally the worst. The unheated air and warm soil condition, Regimes 5 and 6, was consistently good, but was inferior to unheated air and soil in two out of three trials. Based on favorable results obtained under the plastic cover (HC3), they subsequently rooted cuttings achieving humidity control combined with no heating of air or soil in an outside propagation box (Regime 3).

High rooting percentage is achieved with cuttings of loblolly and slash pine at a temperature of 27°C during cooler months and 32°C during the summer (van Buijtenen et al. 1975). The rooting medium is similarly maintained at 27°C.

Soil Temperature

It is well known that adding auxins to the base of stem cuttings generally increases the percentages of rooted cuttings, root counts, and total root weight per cutting. Wells (1985) reported that the efficacy of auxins in promoting the rooting of cuttings depended on the temperature of the medium. This was observed by Hinesley and Blazich (1981) when rooting Fraser fir stem cuttings maintained at two different soil temperatures. IBA alone and wounding + IBA significantly increased the rooting percentage for both rooting medium temperatures, but the improvement was more pronounced at the higher temperature. At the lower temperature, IBA treatments had a negligible effect on the number and length of roots, but these were significantly increased at the higher temperature.

Heating the medium to enhance the rooting of cuttings is a general practice during vegetative plant propagation. Numerous authorities recommend warming the medium to gain a higher percentage of rooted cuttings, faster rooting, and more roots per cutting (McCone 1962, Gislerod 1983). Hartmann et al. (1990) recommended keeping the medium temperature higher than the air temperature to speed cutting root development relative to shoot development. The objective is to induce root activity before shoot growth occurs in the cuttings.

Elevating the temperature of the rooting medium above the air temperature is particularly important when low-pressure mist systems are used for propagation. The relatively low water temperature of the mist combined with the cooling effects of evaporation often result in suboptimal temperatures in the rooting medium. Rapid desiccation of the cuttings or rapid drying of the rooting medium may occur with excessively high temperatures. Therefore, control of the temperature in the rooting medium is essential. Loach (1988) reviewed the methods by which bottom heat can be controlled.

Hinesley and Blazich (1981) observed that visible bud activity was greatest for IBA treated Fraser fir cuttings in the absence of bottom heat, but was considerably less at a higher rooting medium temperature, 20 vs. 24°C, respectively. This decrease in visible terminal bud activity accompanying increased rooting response was unexpected.

Nelson (1966) summarized information on the role of bottom heat in the rooting of cuttings. He concluded bottom heat was more important in the forcing of growing plants than in the rooting of cuttings. Poole and Waters (1971) observed reductions of as much as 50% in time required for propagation of tropical foliage plants during the cool months of the year, when rooting temperature was maintained between 24 and 29°C. Halma (1931) concluded that a rooting temperature of 24 to 26°C was necessary for propagation of softwood cuttings of trees and shrubs. Hamada (1959) emphasized the importance of bottom heat in propagation of mulberry (*Morus alba* L.). Schwartze and Myhre (1947) observed that bottom heat at 21¼°C increased rooting of blueberry (*Vaccinium spp.*) cuttings in March, but was not needed in June.

The study by Bhella and Roberts (1974) showed that rooting temperature had a pronounced effect on

the extent of callus formation on the base of Douglas-fir cuttings. In auxin-treated cuttings, callus formation was fair at 10, good at 18, and excellent at the 26°C rooting temperature, irrespective of SD or LD photoperiod. Rooting temperature did not influence cambial activity in the first year of their two-year study, but it did in year two. While cuttings rooted significantly better under 26 and 18°C temperatures, these temperatures enhanced rooting without affecting bud activity. They concluded that 10°C was too low for optimum metabolic activity and callus formation.

Leakey et al. (1982) conducted two experiments testing the effects of different propagating bed temperatures on root initiation in cuttings of *Triplochiton scleroxylon* K. Schum., an important timber tree of West Africa. The first experiment tested temperatures between 20 and 31°C without auxins, and the second tested temperatures ranging between 23-38°C with auxins. In both experiments, temperatures markedly affected rooting. In experiment I, the percentages of rooted cuttings after week eight increased from zero at 20°C, to 21% and about 36% at 24.5 and 29-31°C, respectively. The rooting percentages were generally greater in experiment II, where the auxin-treated cuttings rooted more rapidly and reached a higher final percentage at 28°C than at 23°C. At higher temperatures (33°C and 38°C) rooting was initially similar to that at 28°C, but cuttings which had not rooted within four weeks started to deteriorate. While approximately 80% of the surviving cuttings rooted in the 28-38°C range, the numbers of survivors at 23°C was significantly greater than at 33°C and 38°C. More roots developed per cutting at 33°C (2.03 ± 0.26) than at 23°C (1.60 ± 0.16).

Raising the temperature from 25 to 30°C increased and accelerated rooting of *Eucalyptus camaldensis* Dehn. cuttings having a relatively intermediate to high rooting capability (Reuveni et al. 1990). Bottom heat did not improve rooting of clones which had low rooting capability. Carville (1979) also observed the rooting of conifer cuttings was promoted by slightly higher temperaures in the base zone.

ROOTING MEDIUM

Cuttings of woody plants are propagated in a variety of media. Reisch (1967) concluded that the particular medium components were of secondary importance in the rooting response, but the resultant physical properties and the management of the medium were of primary concern.

Physical properties of a medium include such parameters as total porosity, bulk density, particle size distribution, air space, water holding capacity, and saturated and unsaturated hydraulic conductivity (Reisch 1967, Hartmann et al. 1990). Of these, aeration and moisture content appear to be the most important properties of a propagation medium. The choice of rooting medium used to propagate conifers and hardwoods is usually by availability, cost, etc. Only a few tests have been specifically designed to evaluate rooting media.

Tilt and Bilderback (1987) conducted a very elaborate study looking at the physical properties of propagation media and their effects on rooting three woody species. Cuttings of a conifer, Leyland cypress (x *Cupressocyparis leylandii* "Haggerston Grey"), a broad-leaved evergreen, holly, (*Ilex* x "Nellie R. Stevens"), and a deciduous tree, crape myrtle (*Lagerstroemia indica*) were inserted in 11 media. The physical properties of seven media were engineered by manipulating particle size distribution of a 1:1 (v/v) aged pine bark:composted hardwood bark medium. Four other propagation media were tested for comparison. With container capacity air space ranging from 12%-40%, and water held after drainage in the root zone ranging from 35%-55%, variation in rooting response of cuttings occurred between species and media, but differences could not be attributed to the physical properties of the various media. Also, no relationship was detected between rooting response and engineered combinations of hardwood bark and pine bark. Only one medium (1 sphagnum peat:1 coarse sand) showed any consistency (within 10%) in rooting between species, and this medium contained the least air space of those compared.

The medium not only influences the percentage of cuttings which form roots, but also the type of root system developed. This observation was tested by Copes (1977) with cuttings from 2- to 4-year old Douglas-fir trees. Twenty-one combinations of sphagnum peat, perlite, vermiculite, and sand in 1:0, 2:1, 1:1, and 1:2 ratios were evaluated. The rooting media yielding the highest percentage of rooted cuttings were: perlite-sand (1:1) - 78%; vermiculite-sand (1:1) - 71%; and sphagnum peat-vermiculite (2:1) - 68%. Large differences in root thickness, flexibility, and branching were noted between cuttings grown in different rooting media.

For Douglas-fir cuttings, Brix and Barker (1973) determined that a mixture of equal volumes of fine peat moss, washed coarse sand, and coarse grade perlite provided favorable aeration, drainage and moisture retention for rooting with the water regime used. The same medium was adopted with favorable results for western hemlock cuttings (Brix and Barker 1975). This is a three component mixture of the same ingredients that Copes (1977) found successful for rooting Douglas fir.

Eliasson et al. (1977) carried out experiments under greenhouse conditions with intermittent mist and supplementary light. Several rooting media were evaluated, but most experiments used sphagnum peat or perlite. Cuttings taken from two-year-old seedlings of Norway spruce throughout the year, rooted well regardless of the rooting media used. The average rooting obtained with this material was 80% .

Cuttings taken monthly (October 1975-September 1976) from 11-year-old Norway spruce rooted poorly in perlite (15% average rooting) and somewhat better in peat (42%). Good rooting was obtained with this material in July in both these media (79% in perlite, 73% in peat) (Eliasson et al. 1977). van Buijtenen et al. (1975) recommended a 50-50 mixture of perlite and vermiculite for rooting cuttings of loblolly pine and slash pine, this was also recommended by Hare (1971).

Leakey et al. (1990) conducted studies using five tree species and investigated the effects of four different rooting media: (ii) gravel, (ii) 50:50 gravel with sawdust, (iii) fine sand, and (iv) 50:50 fine sand and sawdust. Each medium was tested with cuttings dipped in a range of IBA concentrations (0-0.8%). There were substantial differences between species with regard to rooting success on the different media. Single-node juvenile cuttings of all five species rooted well (70-95%) on their best medium. *Cordia alliodora* rooted best in fine sand, with or without sawdust, while rooting of *Vochysia hondurensis* cuttings was detrimentally affected by the incorporation of sawdust into both gravel and fine sand. In contrast, sawdust enhanced rooting of *Eucalyptus deglupta* cuttings in both gravel and sand, while *Gmelina arborea* and *Albizia guachapele* rooted well in all media. The mature cuttings of *G. arborea* did not root as well as the the juvenile cuttings, especially in pure gravel.

Reuveni et al. (1990) successfully rooted cuttings of *Eucalyptus camaldulensis* Dehn. in a wide range of media under intermittent mist. Rooting percentage did not differ significantly among the media used. They concluded that various media could be used for rooting eucalyptus cuttings, as long as the medium contained components that improve aeration. Similar results were obtained in a number of other species when peat, perlite, and vermiculite in various combinations, were components of media producing the higher percentages of rooted cuttings.

Other studies show no significant difference in rooting among media, including Carter (1984) with tamarack (*Larix laricina* [Du Roi] K. Koch) cuttings and Williams (1987) with western white pine (*Pinus monticola* Dougl.) cuttings.

More information is needed about media effects, especially regarding variations in gas-filled pore spaces of substrate without including measurements on other parameters such as mineral nutrients or pH.

MYCORRHIZA

Mycorrhizae formed on the roots of plants by naturally occurring or artificially introduced fungal symbionts are known to enhance the growth and development of their host plants. There is evidence that the fungi increase nutrient uptake in the host plant and produce growth promoting or regulatory compounds (Slankis 1973). Indirect evidence that the fungi release these materials in the host root comes from anatomical and physiological changes in mycorrhizae that can be induced or mimicked by pure preparations of these compounds and/or culture filtrates from the fungi. Therefore, the question is whether ectomycorrhizal fungi produce growth promoting substances that could enhance the rooting of cuttings of vegetatively propagated forest trees.

It is now well established that ectomycorrhizal fungi release IAA (Moser 1959, Ek et al. 1983, Gay and Debaud 1987), which enhances the rhizogenic activity of the host plant and is responsible, at least in part, for the typical morphology of ectomycorrhizae. In different pine species, ectomycorrhizal fungi induce prolific initiation of short roots that are dichotomously branched (Slankis 1973). Long roots are generally nonmycorrhizal but they can be invaded by the symbiotic fungi, which suggests that they are under direct influence of the fungi (Gay 1990).

Linderman and Call (1977) were the first to show that the growth-promoting substances produced by mycorrhizal fungi could promote rooting of woody plant cuttings. Experimenting with 13 different ectomycorrhizal fungi, they demonstrated an increase in the rooting percentage and root volumes of cuttings of bearberry (*Arctostaphylos uva-ursi* L. Spreng.) and huckleberry (*Vaccinium ovatum* Parsh). This stimulation occurred before or in the absence of any mycorrhizal association. Similar results were reported by Navratil and Rochon (1981) on poplar cuttings, and by Branzanti et al. (1985) and Cristoferi et al. (1986) on hardwood cuttings of fruit trees, which are nonectomycorrhizal plants.

Stein et al. (1990) also observed stimulated rhizogenesis of black spruce (*Picea mariana* (Mill.) cuttings by addition of *Laccaria bicolor* (Maire) Orton and *Suillus cavipes* (Opat.) Smith & Thiers to the rooting medium over controls, although the best rooting percentage and highest number of roots was formed on auxin-treated cuttings. The results observed with European larch (*Larix decidua*) cuttings were different, in that the two fungal symbionts had no significant effects on rooting. No ectomycorrhizal formation occurred in *S. cavipes* inoculated medium with either tree species.

Gay (1990) studied the effect of the ectomycorrhizal fungus *Hebeloma hiemale* Bres. and its culture filltrate on in vitro rooting of Aleppo pine (*Pinus halepensis* Mill.) isolated shoot hypocotyls to determine if ectomycorrhizal fungi could enhance adventitious root formation. *P. halepensis* hypocotyls did not root in the absence of hormonal treatment, whereas the rooting percentage was 87.3% in the presence of 5 μM IAA. In the presence of Trp (tryptophan), which is a precursor of IAA, *H. hiemale* strongly enhanced rooting of hypocotyls cultivated in the absence of any hormonal treatment. In the presence of 0.1 mM Trp, the rooting percentage of the inoculated hypocotyls was 96.6%, whereas it was only 7.6% in the absence of the fungus. *H. hiemale* culture filtrate obtained in the absence of Trp did not contain IAA and did not stimulate rooting of the hypocotyls. In contrast, a culture filtrate obtained in the presence of Trp contained IAA; an ethyl acetate extract from this filtrate resulted in 100% rooting. Different fractions were isolated by thin-layer chromatography from the IAA-containing filtrate and studied for their effect on rooting. It was demonstrated that IAA was responsible for the rhizogenic activity of *H. hiemale*. These results suggest that ectomycorrhizal fungi which rapidly metabolize exogenously supplied Trp to IAA could be a suitable tool to enhance rooting of gymnosperms. They also confirm previous reports (Libby and Conkle 1966, Bowen et al. 1975, Smith and Thorpe 1975) that rooting of gymnosperm cuttings (e.g. radiata pine [*Pinus radiata* D. Don] or lodgepole pine [*Pinus contorta*]) can be enhanced by auxins. It must also be emphasized that ectomycorrhizal fungi also release cytokinins, gibberellin-like compounds, and ethylene (Gay 1988).

Mechanism of Effect

Several mechanisms could be operating with this enhanced rooting response. The rooting effect of these fungi is probably due to a nonspecific mehanism and could be ascribed to growth regulators (e.g. auxins, cytokinins, ethylene or gibberellins), vitamins, or other metabolites released by the fungi (Slankis 1973, Gay 1990). It can also be postulated that inoculation of cuttings with these fungi could modify their hormonal balance and subsequently their rooting ability. Navratil and Rochon (1981) suspected hormonal exudates liberated by the mycelium of *Pisolithus* as a cause of the enhanced shoot and root development in poplar cuttings.

Other possible mechanisms for the rooting phenomenon include the mycorrhizal fungi producing substances which may act protectively or synergistically with the growth substances already in cuttings (Linderman and Call 1977). Levisohn (1956) suggested that these fungi may be altering the nutrient status of the medium, or may be releasing substances that are inhibitory to other microbes that cause cuttings to deteriorate.

Endomycorrhizal Inhibition

Endomycorrhizal colonization has also been shown to stimulate growth of greenhouse-grown seedlings of hardwood species (Kormanik and McGraw 1982, Melichar et al. 1986). However, there are results which show that the potential benefits associated with endomycorrhizal inoculation were not expressed in plant growth measurements of cuttings during the root production phase.

Sword et al. (1991) conducted an experiment to identify the effect of inoculation using a mix of three *Glomus* spp. isolates on the root growth rate of eastern cottonwood (*Populus deltoides* Bartr. ex Marsh.) cuttings. Results indicated that endomycorrhizal inoculation appeared to have a negative effect on growth during greenhouse

production. There was no significant effect on stem length, stem diameter, or on stem, root, or foliar dry weights of the cuttings. The leaf surface area of inoculated cuttings was significantly less than that of uninoculated cuttings. Shoot and root growth were either unaffected or inhibited due to endomycorrhizal inoculation.

Sword et al. (1991) attributed negative root growth rate and leaf surface area responses to a combination of factors. They suggested that endomycorrhizal inoculation may have affected root system morphology. Also, the physiology of hardwood cuttings may provide some explanation for the growth inhibition observed. Nanda et al. (1971) reported the importance of having adequate exogenous glucose, in addition to IAA, for rooting of etiolated *Populus* cuttings. It has been reported (Tschaplinski and Blake 1990) that a proper balance of nutritional and regulatory compounds determines the rooting ability of this genus. The rapid rate of shoot growth of cuttings as compared to seedlings suggests that starch availability for initial root growth may be more limiting in cuttings than in seedlings. Furthermore, the shoot growth rate of cuttings compared to seedlings suggests that production of growth regulators in shoot meristematic tissues may be greater in cuttings. As a result, growth responses of cuttings and seedlings inoculated with endomycorrhizal fungi may differ.

Another explanation for the negative effect on growth is that a limited availability of photosynthate for root growth, due to higher metabolic requirements of plants with endomycorrhizal fungal associates than without, may have contributed to reduced growth of the host. Other factors which may help to explain the unexpected decrease in growth of inoculated cuttings in this particular study, may involve competition between the three endomycorrhizal isolates, in combination with low greenhouse light conditions.

THE ROOTING FACILITY

Container (Enclosed) vs. Open Mist Beds

The appropriate choice of propagation facility is based on the level of humidity and the amount of temperature control that is required to root a particular species, with enclosed beds offering more control over these factors. Sensitivity of the plant's tissues to varying moisture levels, is another consideration. Both systems are usually shaded which is essential in most systems to keep water stress to a minimum (Loach 1977). It has been shown that mist inside a closed polyethylene tent reduced the leaf-to-air vapor pressure gradient more effectively than conventional open mist beds (Grange and Loach 1983).

Grange and Loach (1984) compared rooting of 81 species of leafy cuttings in open and polyethylene-enclosed mist systems. Enclosing a mist system in a polyethylene tent affected the environment and cuttings in several different ways. Because the leaf surfaces were wetter and the humidity greater under enclosed mist, water loss from the cuttings was reduced and the water potential of the foliage was significantly greater (less negative) than under open mist. Light levels were 20% lower and air temperatures averaged 5°C higher in enclosed mist than in open mist.

A comparison of rooting performance in the enclosed mist and open mist systems suggests that the enclosed mist system would be preferable for the propagation of cuttings susceptible to water stress, though physiological principles show open systems are likely to be superior for net photosynthetic gain, but inferior in terms of water stress (Grange and Loach 1984). Other factors such as leaf wetness, which can cause rotting, must also be considered.

Greenhouse vs. Nursery vs. Field Rooting

In propagation procedures, cuttings are sometimes planted directly in the nursery bed or field, but most often they are started in a blended mix in some type of container. Rooting in these three environments is segregated by the degree of environmental control available within each system.

- Conventional greenhouse rooting can usually be designed so that humidity, temperature, light, and other parameters (e.g. CO_2 enrichment) can be either precisely controlled or relatively controlled. The cuttings are stuck into individual rooting containers or into rooting beds. Humidity is controlled with fog or ultra-low volume mist. Temperature inside the greenhouse can be controlled by heaters, evaporative coolers, exhaust fans, and movable side walls or vents.

- A covered nursery bed is usually a plastic-covered metal frame installed over several nursery beds. Humidity and temperature, based on whether

beds are located indoors or outdoors, can be controlled with this system.

- Field rooting allows for no environmental control. Water/fertilizer is the only element of the environment that can be provided to cuttings, generally through some type of drip irrigation system.

The high cost of plantation establishment dictates that the very best planting stock be used. Methods to produce rooted stock have met with variable success. Many times the decision on which propagation facility to use has been a purely economic rather than a practical decision based on efficiency of the method. Most often, foresters have met less success by adopting methods for establishing and managing large plantations than those accomplished with cottonwood cuttings (McKnight 1970, Randall and Miller 1971). Under ideal circumstances, cottonwood performs as no other tree species can.

The lack of success has prompted the investigation of potential methods of improving rooting ability and ease of propagation to acceptable levels for commercial production. Cunningham (1986) evaluated the rooting ability of cuttings from one-year-old coppice of American sycamore for 80 ortets (4 trees from each of 20 half-sib families) in a greenhouse and a nursery environment. Rooting percentages averaged 97.26 for the greenhouse and 56.29 for the nursery study. Greenhouse rooting success was excellent for all but a few clones, and nursery rooting, while not as high as greenhouse, was encouraging. The quality of the nursery-rooted cuttings and the number of clones with good rooting success (20% of the clones rooted at 80% or better) indicated that it was feasible to produce American sycamore from nursery-rooted planting stock.

Findings were inconclusive in a study conducted by Foster (1978), in which he compared the rooting behavior of loblolly pine stem cuttings in an open medium, and in 164-ml plastic Ray Leach Supercell containers, both under mist. Rooting success was 39 and 24%, respectively, showing no significant differences. Although there was a large degree of variability in this experiment, there were some advantages to using containerized cuttings, including easier and quicker rooting observation, and less damage during lifting of cuttings rooted in the open medium.

While the cost of rooting and growing large numbers of planting stock in the greenhouse may be prohibitive, particular clones of high value for growth and wood properties "may" justify the added expense of nursery rooting. Clonal selection for nursery rooting ability would probably place most emphasis on improving percent rooting, as opposed to other selection traits (Cunningham 1986). Higher rooting percentages would save money with less area needed for stock plants and smaller nursery requirements to produce the same quantity of planting stock. Greenhouse rooting, with its high rate of success, could be used in the initial stages of multiplying the number of stock plants for mass production of cutting material.

CONCLUSIONS

Based on the potential impact in forestry that vegetative propagation by rooted cuttings can have, the search for more efficient methods to produce operational planting stock must continue. Production of quality propagules, similar to those which are routinely achieved for Japanese cedar (*Cyptomeria japonica* [L.F.] Don), Norway spruce, radiata pine, and eastern cottonwood, by rooted cuttings is anticipated in the future. Many of these trees have been growing under plantation conditions sufficiently long to demonstrate the success of clonal stock and provide impetus to solve the problems of difficult-to-root genera and species. Other important commercial forest species, such as loblolly pine, slash pine, and Douglas-fir are in various stages of development to support a production program for forest plantations.

This review shows that there is incomplete understanding of the various interactive factors involved in providing optimal environmental conditions for the rooting of cuttings from forest trees, and the results from some prior studies have often raised difficult questions. The purpose of studies cited in this paper was to better understand the processes underlying improved rooting by following what is documented about humidity, water relations, light, air and soil temperature, the rooting medium, mycorrhiza, and the rooting facility.

Optimal conditions encourage further development towards fully operational clonal forestry. Rigorous control of the rooting

environment promises to improve the success of rooting larger numbers of cuttings. More studies are required to understand exactly how ectomycorrhizal fungi affect rhizogensis. However, the ability of the fungi to enhance rooting of coniferous cuttings in absence of any auxin supply has been demonstrated, and may represent an alternative to the use of auxins for the vegetative propagation of conifers from cuttings.

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Propagule Growth, Development and Application

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FIELD PERFORMANCE COMPARISONS BETWEEN VEGETATIVE PROPAGULES AND SEEDLINGS OF LOBLOLLY AND SLASH PINES[1]

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Abstract.--Current knowledge of the field growth of loblolly and slash pine tissue culture propagules and rooted cuttings relative to seedlings is reviewed. Generally, tissue culture propagules produced via axillary- fascicular micropropagation from epicotyls and rooted cuttings from juvenile ortets perform similarly to seedlings under field conditions. Tissue culture propagules produced via the Cotyledon System and rooted cuttings from older ortets generally express mature characteristics including reduced growth relative to seedlings. The knowledge and ability to manipulate the degree of planting stock maturation will be required in order to effectively develop operational vegetative propagation programs for these species.

INTRODUCTION

Currently, numerous forest products companies in the Southern U.S. are interested in developing commercial vegetative propagation systems for loblolly pine (*Pinus taeda* L.), slash pine (*Pinus elliottii* var. *elliottii* Engelm.) or both. Their incentive is to capture the benefits associated with deploying reforestation planting stock either of elite control-pollinated crosses and/or clones.

Often, it has been assumed that vegetative propagules will perform similarly to seedlings in the field. However, the process of vegetative propagation, or even factors unique to a specific method of vegetative propagation, may cause the resulting propagules to behave dissimilarly to seedlings. In order to fully capture the benefits of this technology, an understanding of and ability to manipulate any differences between vegetative propagules produced via various methods and seedlings is necessary.

The objective of this manuscript is to review the current knowledge of field performance of loblolly and slash pine vegetative propagules relative to that of seedlings.

TISSUE CULTURE PROPAGULES

Loblolly Pine

Three methods of tissue culture propagation of loblolly pine have been summarized: 1) organogenesis of adventitious shoots and roots from cotyledon explants (Cotyledon System), 2) micropropagation via fascicular and axillary shoots obtained from juvenile, adolescent and mature explants and 3) somatic embryogenesis using immature zygotic embryos as a starting source (Amerson et al. 1988, Greenwood et al. 1991).

The current knowledge of the field performance of propagules produced from each of these tissue culture methods is reviewed below.

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Cotyledon System.--Establishment of field trials comparing loblolly pine Cotyledon System propagules and seedlings began in the late 1970's and early 1980's so that field performance of propagules produced in this manner has been studied intensively.

Growth and Survival.--While Cotyledon System propagules generally survived field planting as well as seedlings, they displayed reduced early height and diameter growth relative to seedlings (Amerson et al. 1985). For example, in a series of eight field trials established during 1981, the mean fourth year height of these propagules was significantly less than that of seedlings from the same open-pollinated families (4.40 versus 5.06 m, respectively) (Amerson et al. 1988, Frampton 1986). Large differences in early diameter growth between these Cotyledon System propagules and seedlings were also found (Mott et al. 1984). Measurements through year eight in one of the 1981 trials located near Monroeville, Alabama, verified that this trend continued into later years (Mott et al. 1990). However, propagule types in these studies were established in paired row-plots so that measurements subsequent to crown closure were confounded with competition effects. Nevertheless, it is clear from these and other studies that Cotyledon System propagules grow considerably slower than seedlings.

Shoot Morphology.--Differences in shoot morphology between Cotyledon System propagules and seedlings were often casually observed. A detailed assessment of various characteristics which change with maturation in loblolly pine (Greenwood, 1984) (bud size, number of growth cycles, number of branches, etc.) after two growing seasons at one field trial in Jesup, Georgia, verified quantitatively that Cotyledon System propagules produce more mature-like morphology relative to seedlings of the same open-pollinated families (McKeand 1985). A later set of measurements in the same trial after four growing seasons and in a Raleigh, North Carolina, trial after six growing seasons revealed that these morphological differences between propagule types lessen at older ages (Mott et al. 1987).

Fusiform Rust Resistance.--Generally, Cotyledon System propagules have shown less fusiform rust (caused by *Cronartium quercuum* (Berk.) Miyabe ex Shirai f. sp. *fusiforme*) infection than have seedlings. In five of the eight field trials established in 1981, rust infection levels exceeded 60%. The Cotyledon System propagules in these five trials had a lower incidence of gall formation at age four than did seedlings from the same open-pollinated families (46 versus 71%, respectively) (Frampton 1986).

Wood Specific Gravity.--Juvenile wood specific gravity of Cotyledon System propagules and seedlings were compared at three field trial locations each of a different age: Lewiston, North Carolina (age two), Jesup, Georgia (age three) and Raleigh, North Carolina (age six) (Frampton and Jett 1989). A significant difference in the mean specific gravity between propagule types was found only in the youngest material (0.387 for the seedlings versus 0.356 for the cotyledon system propagules.

Root Systems.--Cotyledon System propagule root systems were generally less developed than those of seedlings. Under greenhouse culture they typically produced less root dry weight and fewer lateral roots in the upper portion of their root systems than did seedlings (McKeand and Allen 1984, Frampton and McKeand 1987). The main differences between Cotyledon System propagule and seedling root systems appeared to be morphology rather than physiology since their ability to uptake nutrients was similar based on root surface area (McKeand and Allen 1984). Root excavations of Cotyledon System propagules and seedlings confirmed that these morphological root system differences persist after 2.5 years in the field (Frampton and McKeand 1987).

Cause of Differences.--Differences between Cotyledon System propagules and seedlings were recognized early so that in 1982, two field trials were established to better understand these differences (Frampton and Isik 1987). Both of these field trials contained Cotyledon System propagules and seedlings from the same open-pollinated families as well as two additional in vitro-produced propagule types: excised embryos and rooted hypocotyls. As in other field trials, after 3.5 growing seasons, the Cotyledon System propagules were shorter in height, had less fusiform rust incidence and displayed more mature morphology relative to the seedlings. The two additional propagule types performed similarly to the seedlings for all traits assessed. Designed comparisons among these four types of propagules suggest that the origin of differences between Cotyledon System propagules and seedlings lies with in vitro shoot production methodology rather than with the tissue culture environment or rooting procedures.

Another field trial designed to understand differences between Cotyledon System propagules and seedlings was established in late 1983 (Anderson et al. 1992). After one growing season, scion of Cotyledon System propagules and seedlings were grafted onto rootstock of Cotyledon System propagules and seedlings of the same open-pollinated families so that four grafted combinations were obtained. Three growing seasons after grafting, Cotyledon System roots accounted for 0.3 m of height growth loss and 1.0 cm of basal diameter growth loss while the Cotyledon System shoots accounted for 0.6 m of height growth loss and 1.4 cm basal diameter growth loss. The roots of Cotyledon System propagules used in this study were initiated and developed for 18 months prior to grafting under the control of Cotyledon System shoots. This may account for the contribution of the Cotyledon System roots to growth reduction in this trial that was not apparent in the earlier pair of trials (Frampton and Isik 1987). As in the earlier pair of trials, three years' data in this trial indicated that the expression of mature shoot characteristics of Cotyledon System propagules is controlled almost entirely by their shoots rather than roots.

One additional field trial was established in 1988 to understand differences between Cotyledon System propagules and seedlings (Frampton et al. 1992). In this trial, seedlings, Cotyledon System propagules, axillary-fascicular micropropagules originating from epicotyls (see below) and axillary-fascicular micropropagules originating from Cotyledon System shoots were established. Based on measurements assessed during the fifth growing season, the height and diameter at breast height (DBH) of the Cotyledon System propagules and the micropropagules derived from Cotyledon System shoots were significantly less than that of the other propagule types which were similar to each other. Again, these results suggest that the method of adventitious shoot production from cotyledons results in dissimilarities from seedlings in the propagules produced. Further, these differences are maintained through up to three cycles of micropropagation.

Axillary-Fascicular Micropropagation Juvenile Sources.--One field trial containing axillary-fascicular micropropagules of loblolly pine was established near Cantonment, Florida, in 1988 (Frampton et al. 1992). As mentioned above, this field trial contained axillary-fascicular micropropagules originating from epicotyls, seedlings, Cotyledon System propagules, and axillary-fascicular micropropagules originating from Cotyledon System shoots. The micropropagules originating from epicotyls were also produced using three levels (0, 25 and 50 mg/l) of benzylamino-purine (BAP) in the shoot initiation procedure. Unlike the cotyledon-origin adventitious shoots, the micropropagules in this field trial grew similarly to the seedlings based on first and fifth year height and diameter assessments. Their first year shoot morphology was also similar to seedlings. Further, the three levels of BAP used for shoot initiation did not have any significant effect on the subsequent growth and development of these micropropagules.

Adolescent and Mature Sources.--Although techniques exist to propagate adolescent and mature loblolly pine via axillary-fascicular micropropagation (Amerson et al. 1988), no field performance data have been reported. Such propagules would be expected to express some degree of rejuvenation. Their rate of maturation relative to seedlings would be of great interest. Data from such trials could contribute greatly to the current understanding of maturation and rejuvenation.

Somatic Embryogenesis.--Currently, intensive research efforts are focused on developing this technology as a potentially more productive tissue culture system for loblolly pine. Although a small number of loblolly pine somatic seedlings have been field-planted to date, no reports of their field performance currently exist in the literature.

Slash Pine

Although the same methods of tissue culture propagation reported for loblolly pine could be applied to slash pine, considerably less research has been conducted on this species. In one field trial established near Yulee, Florida, slash pine Cotyledon System propagules were compared to seedlings. Three-year-results showed a similar but smaller difference in height growth between these propagule types than was observed for loblolly pine.

No reports of the growth of slash pine propagules produced via other tissue culture methods currently exist.

Rooted Cuttings Loblolly Pine

Growth.--Growth of loblolly pine rooted cuttings was compared with that of seedlings in two field

trials established in central Arkansas during 1978 and 1980 (Foster et al. 1987). In the older trial, rooted cuttings from four-year-old ortets were significantly smaller in height ($P \leq 0.05$) and DBH ($P \leq 0.01$) at age six than were seedlings from the same open-pollinated families. In the younger trial, cuttings from one-year-old ortets were significantly ($P \leq 0.01$) larger in height and DBH than rooted cuttings from five-year-old ortets and seedlings from the same open-pollinated families. The mean height and DBH of the rooted cuttings from five-year-old ortets and seedlings were not significantly different from each other. However, the seedlings in this trial were one growing season younger and considerably smaller in size than the rooted cuttings when the trial was initially established. The seedling height growth curve was parallel to that of the rooted cuttings from one-year-old ortets and steeper than that of cuttings from the five-year-old ortets.

Together, the results from these trials demonstrate the negative effect of increased ortet age on the growth of loblolly pine rooted cuttings and suggest that the early field growth of rooted cuttings of one-year-old ortets and seedlings is similar. Results from a smaller field trial comparing rooted cuttings from young ortets with seedlings of the same five control-pollinated families corroborates this conclusion (Foster 1988). Height and DBH after three years in the field were not significantly different for the rooted cuttings and seedlings in this trial.

Shoot Morphology--Shoot morphology characteristics were measured in the three studies discussed above (Foster et al. 1987, Foster 1988). Differences between rooted cuttings and seedlings were not consistently found. However, when differences were detected, the rooted cuttings displayed less stem taper, fewer growth cycles and fewer branches than did the seedlings.

Fusiform Rust Resistance--No loblolly pine rooted cutting field trials with fusiform rust infection have yet been reported. However, two studies utilizing artificial inoculation and disease development of greenhouse-grown containerized seedlings and rooted cuttings have been conducted.

In the first study (Foster and Anderson 1989), full-sib families of seedlings were inoculated and symptoms were assessed using standard protocol (Anderson et al. 1983). After symptom assessments, those seedlings without visible gall formation were developed into hedges. Cuttings from these hedges were rooted, inoculated and assessed using the same protocol as for the seedlings.

In this study, the mean percentages galled for the rooted cuttings and seedlings were approximately 4 and 53%, respectively. The range in seedling and rooted cutting family means were 41-64% and 0-11% galled, respectively. Further, a resistant and susceptible seedling checklot included with the rooted cutting inoculations averaged 50 and 80% galled, respectively.

While the results from this study showed a dramatic difference in fusiform rust resistance between seedlings and rooted cuttings, the following confounding factors make it impossible to conclude to what degree, if any, that this difference is due to inherent differences between propagule types: 1) the cuttings had been screened for resistance while the seedlings had not; and 2) succulent epicotyl tissue was inoculated on the seedlings while second year growth was inoculated on the rooted cuttings.

In the second study (Frampton and Walkinshaw 1988, Unpublished Data), clones of cuttings along with seedlings from the same 14 open-pollinated families were inoculated together using standard protocol (Anderson et al. 1983). In this study, the rooted cuttings were sheared and new fascicular shoots were allowed to develop so that the inoculated tissue was in a similar stage of development. Additionally, there was no differential selection for resistance between the rooted cuttings and seedlings.

Results of this study also revealed a marked difference in resistance between the rooted cuttings and seedlings: 19 versus 43% galled, respectively. These results suggest that only a portion of the difference in propagule type resistance reported in the first study was a result of screening for resistance. While phenological differences between rooted cuttings and seedlings cannot be ruled out in the later study since there are differences between growth and development of fascicular shoots versus epicotyls, it appears that there may be a large degree of resistance associated with using rooted cuttings rather than seedlings as planting stock. Ultimately, rooted cutting and seedling comparisons must be made under field conditions on high rust hazard sites to corroborate this conclusion.

Slash Pine

Growth.--An early report (Hoeskstra and Johansen 1957) suggested that ortet age influenced subsequent growth of vegetative propagules of slash pine. Although seedling comparisons were not included, air-layers from six-year-old ortets were substantially taller than those from 23-year-old ortets after 1 year in a nursery. In another study without seedling comparisons, slash pine air-layered from the main stem and first-order branches of four-year-old ortets were reported to grow similarly (Greene 1962).

In a later reported study established from 1944 to 1947 (Franklin 1969), the effect of ortet age on growth was quantified. Height and DBH measurements of 21- to 24-year-old rooted cuttings were regressed on ortet age (ranging from 12 to 28 years). The regression equations suggested that each ortet year reduced height and DBH of the rooted cuttings by about 12 cm and 39 mm, respectively. In another field trial involving air-layered and grafted material, similar relationships were also found (Franklin 1969).

A planting of slash pine air-layers from 11-year-old ortets was established in 1957 and 1958 in northeast Florida near Olustee (Schultz 1972a and b). This trial was designed as a split-plot factorial to study the effect of various cultural treatments of oleoresin production. The plots in the study were separated by one or two rows of commercial seedlings.

While the fastest growing air-layered clone had three times the merchantable stem volume as the slowest growing clone at age 12, the overall mean merchantable stem volume of the air-layers was almost identical to and not statistically different from that of the seedlings (Schultz 1972a). The mean height and DBH of the air-layers were slightly greater that those of the seedlings after 30 years: 21.8 versus 22.8 m and 32.6 versus 34.7 cm, respectively. Inspection of DBH (inside bark) growth curves reconstructed from increment core measurements revealed that during certain periods of the rotation, the air-layer mean diameter equaled or exceeded that of the seedlings. These differences in growth between the air-layers and seedlings were slight, however, when compared to the variation among the clones of air-layers: 18.9-24.3 m in height and 22.9-41.2 cm in DBH at age 30.

The similar growth of the propagule types in this study, despite the relatively old air-layer ortet age, may be attributed to two factors: 1) air-layers are likely to be of much better vigor with more profuse root systems than rooted cuttings due to the benefits of rooting attached to the parent tree and 2) the air-layers were likely taller at establishment since slash pine air-layers were typically up to 60 cm in length (Mergen 1955). However, the results from this field trial suggest that it may be possible to reverse the negative effect of ortet age on growth by providing special care to improve the planting size, vigor and root system of vegetative propagules from older ortets.

Root Systems.--Root development of 11- to 12-year old air-layers (from 11-year-old ortets) and seedlings were compared in the field trial mentioned above which was established on a moderately deep sandy soil (Schultz 1972b). The air-layers and seedlings did not differ significantly in total root surface area or in shoot growth (see study discussed above, Schultz 1972a). However, all seedlings developed well-defined tap roots with primary lateral roots at depths of 0 to 1.5 m, while most air-layers had one to three heavy sinkers arching from the root collar to the maximum root depth which sometimes developed lateral roots. The average depth to which roots developed were 2.3 and 2.6 m for air-layers and seedlings, respectively. Although not statistically significant, seedlings averaged 44% more primary lateral roots and 79% more sinker roots than did air-layers. The average root diameter differed only slightly between the two propagule types. The root systems of individual trees varied greatly except that members of air-layer clones were remarkably similar to each other.

GENERAL DISCUSSION

Generally, vegetative propagules of loblolly and slash pine survive and grow normally under typical field conditions and do not exhibit gross aberrations. No reports of plagiotropic field growth of slash and loblolly pine vegetative propagules exist. While both tissue culture propagules and rooted cuttings of these species commonly display plagiotropic growth in the greenhouse, apparently, under field conditions, this response is corrected by the production of compression wood.

When differences between vegetative propagules and seedlings have been reported, the vegetative

propagules displayed reduced growth, mature shoot morphology or both. While reduced growth is undesirable for commercial forestry applications, many of the mature characteristics such as reduced fusiform rust infection, less stem taper and fewer branches are highly desirable characteristics. The ortet age at which the tradeoff between fast growth and desirable shoot characteristics occurs is not known for loblolly and slash pines. More data regarding the influence of ortet age on the growth of rooted cuttings are available for Monterey pine (*Pinus radiata* D. Don). In this species, rooted cuttings from young ortets (one- to three-years-old) perform at least as well as seedlings. Rooted cuttings from slightly older ortets (four- and five-year-old) display some loss of early diameter growth but have better form with less malformation (Menzies et al. 1991).

Commercial rooted cutting programs designed to bulk-up elite control-pollinated families or clones will require the use of hedges and/or serial propagation. These techniques are reported to slow down the maturation process in other species. However, for loblolly and slash pine, little is known about this process, especially with relation to subsequent field performance.

Obviously, further field trials are needed to provide the knowledge necessary to develop effective vegetative propagation systems for loblolly and slash pine. While not emphasized in this review, many of the past field trials comparing vegetative propagules and seedlings have various shortcomings. The following recommendations are provided to help circumvent some of these shortcomings in future field trials of this type (see Frampton and Foster 1992 for further details): 1) compare propagule types of similar genetic quality such as from paired open-pollinated families, 2) make every effort to culture the propagule types in a manner so that they can be planted at the same time and be of comparable size and physiological condition, 3) use block plots for propagule types rather than row or single tree plots in order not to confound competition with propagule type effects after crown closure and 4) measure the trials at early ages and frequent intervals.

CONCLUSION

Vegetative propagules of loblolly and slash pines often perform similarly to seedlings under field conditions. When this is not the case, vegetative propagules generally display reduced growth and mature-like morphological characteristics. Better understanding how to control these differences will be necessary in order to effectively utilize vegetative propagation technology in the future and may become a key advantage of using this technology.

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APPLIED VEGETATIVE PROPAGATION PROGRAMS IN FORESTRY[1]

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Abstract.--Since the 1970's there has been a surge in the use of vegetative propagation and reforestation on a commercial scale. The genetic improvement strategies and methods of vegetative propagation are reviewed with particular emphasis on the two largest programs in the United States, one at James River Corporation and the other at Weyerhaeuser Company. The benefits, problems and limitations in the implementation of vegetative propagation on a commercial scale are discussed.

INTRODUCTION

Before the 1970's very few commercial applications of vegetative propagation existed in forestry. A notable exception is the centuries old program of clonal plantations of sugi (*Cryptomeria japonica* D. Don) in Japan (Ohba 1983). Eucalypts, radiata pine (*Pinus radiata* D. Don) and Norway spruce (*Picea abies* [L.] Karst) have been regenerated by rooted cuttings since the 1970's, and during the 1980's efforts have expanded to several other species. In the past 20 years there has been a surge in the number of organizations worldwide that use vegetative propagation on a commercial scale. Survey results indicate that at least 70 million conifer cuttings are produced annually in 20 countries (Ritchie 1992a). We are unaware of similar survey results for hardwoods but worldwide production of hardwood rooted cuttings is probably more than twice that of conifers. The largest programs are in Brazil, The Congo and South Africa where eucalypts are planted in monoclonal plantations (Brandao 1984, Delwaulle 1985, Denison 1989). Vegetative propagation as a proportion of total regeneration is expected to grow considerably in the coming decades and may become the norm for many species which are now being regenerated by seedlings.

There are a number of publications on operational vegetative propagation programs, besides those already mentioned to which the reader is referred (Armson et al. 1980, Arnold and Gleed 1985, Bentzer 1981, Foster and Shaw 1987, Gill 1982, Johnsen 1985, Kleinschmidt 1974, Lambeth et al. 1992, Ritchie 1992b, Zobel 1992). A complete review of operational programs worldwide will not be attempted here. For a synopsis of international regeneration activity with conifer rooted cuttings the reader is referred to a recent article by Ritchie (1992a). Emphasis is given here to: 1) benefits of rooted cuttings which make them so attractive to many organizations, 2) tree improvement and propagation systems being employed, and 3) problems and limitations of rooted cutting production. Two large-scale, vegetative propagation programs in the United States are used as case studies.

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POTENTIAL BENEFITS OF VEGETATIVE PROPAGATION

At this time all applied vegetative propagation programs in forestry use rooted cuttings though some promising research continues on micropropagation systems, the most promising of which is somatic embryogenesis. These programs began for various reasons depending on the species, final product and ease of propagation. Some of the more commonly cited reasons and benefits follow.

Gains in Growth Rate

Most programs are aimed at the production of clonal plantations or multiplication of high value full- or half-sib families from seed orchards or hybrids. Potential gains in yield vary greatly from species to species but can be substantial when compared to the best available seedlings. Most conifer programs are aimed at bulking up seed of seed orchard genotypes. Notable exceptions are sugi in Japan and Norway spruce in southern Sweden where emphasis is placed on the testing and deployment of clones. By contrast, all hardwood rooted cutting programs are aimed at the production of clones. This is due to their ease of propagation and absence of serious stock plant maturation problems. Detailed systems of propagation and tree improvement options are covered in a later section.

Maturation of stock plants can negate or reduce potential genetic gains in growth. Cuttings from mature stock plants often exhibit poorer rooting, slower growth and morphological abnormalities such as plagiotropism as compared with their counterparts from juvenile stock plants or as compared to seedlings of equal genetic value.

Improved Quality

Some rooted cutting programs have been justified primarily on improved quality of the final product, especially in radiata pine (Arnold 1985, Spencer 1987). Apparently, cuttings maintain some of the mature qualities of older stock plants resulting in fewer and smaller branches and straighter stems. In some programs, expectations in growth rate gains may be nominal and, in other cases, there is an acceptance of reduced growth of rooted cuttings in favor of their higher quality. The clonal eucalypt program at ARACRUZ has made impressive volume-per-hectare gains of over 50% over nonimproved seedlings, but testing for specific gravity and pulping qualities is also a crucial part of the program (Brandao 1984).

Speed of Propagation of High Value Genotypes

One of the most commonly cited reasons for the use of rooted cuttings is very slow seed production in orchards, especially in species such as Norway spruce in northern Scandinavia and black spruce (*Picea mariana* [Mill.] B.S.P.) in Canada (Armson 1980, Gill 1982, Johnsen 1985, Ritchie 1992a, 1992b). Small quantities of seed from the best open-pollinated or full-sib families are multiplied to commercial quantities. In many areas of the tropics, especially near the equator, exotic conifers may produce little to no seed (Gallegos 1983, Dvorak and Lambeth 1992) and vegetative propagation may be necessary, or at least a financially attractive alternative to producing seed.

Reduced System Cost

Rooted cuttings of conifers typically cost two to three times that of seedlings when one looks at only the nursery phase. However, it is invalid to look at only one phase of propagation. One option now being explored is the possibility having only small seed orchards produce small quantities of seed which can be multiplied by rooted cuttings. The cost of such a system may be less than operating a full-scale orchard and conventional nursery for seedling production. Some of the economic gains from planting rooted cuttings will be offset by higher propagation costs using current rooting techniques, but more inventive propagation systems and more cost-effective rooting procedures will certainly change this situation.

James River Corporation roots cuttings of poplars directly in commercial plantations with no need for a nursery, thereby achieving lower regeneration costs than those possible with conventional seed orchards and nurseries. ARACRUZ has found rooted cuttings to be less expensive than seedlings of *Eucalyptus grandis* (Hill, ex Maiden) and its hybrids because the time cuttings spend in the nursery is less than that for seedlings which reduces the necessary nursery space (Dr. Bruce Zobel, Zobel Forestry Associates, pers. comm.). The savings are substantial because ARACRUZ plants year-round. In the beginning of rooted cutting research and early operations, the system savings were largely overlooked but, in the future, more rooted cutting programs will be started when the decision of

whether or not to establish a new orchard becomes necessary.

Uniformity and Predictability of Improved Stock

In established programs which produce monoclonal plantations of eucalypts and sugi, crop uniformity is cited as an advantage (Brandao 1984, Lambeth 1992, Ohba 1985). In the case of eucalypts, the predominant uniformity advantage is uniform wood which can be processed more efficiently and which produces a more uniform pulp. In some programs only about 20 tested clones, out of thousands tested, can be used for regeneration; while in seed orchard programs it is common to regenerate with only a few families selected from a few hundred tested. In the case of a clonal program, it is much easier to find 20 clones of similar wood qualities due to the high selection intensity than it would be in the seed orchard program where selection is lower and wood quality varies among individuals within a family.

Hypothetically, uniform clonal plantations should also have less inter-tree competition and, therefore, less competition-induced mortality than seedling populations. Detailed studies have not yet proven this hypothesis, but one can observe more late mortality in stands from seed than in clonal plantations.

Seed orchard families, especially open-pollinated families from the first generation, are highly variable, and their superiority over unimproved seed lots is often difficult to observe and may be unpredictable under field conditions. As tree improvement programs mature, geneticists will find it more and more important to provide commercial genotypes which are visually superior in a commercial setting in order to justify continued existence of decades-old programs. More uniform genotypes will help make this possible.

Improved Survival?

Although there has been great concern about the quality of rooted cutting root systems and their field adaptability, most studies indicate that, when overall rooting percent is acceptable, cuttings survive as well or, in some cases, better than their seedling counterparts (Lambeth et al. 1992, Ritchie 1992a, Struve and McKeand 1990). Lambeth et al. (1992) observed statistically significant higher mortality in seedlings than in rooted cuttings of *Eucalyptus grandis* in the first year with continued higher mortality in the seedlings in the second and third years. Seedling check lots consistently ranked low for survival in 16 clonal trials. By the third year the difference was 11% (83% survival for seedlings versus 94% for clones), a statistically and operationally significant difference. Ritchie et al. (1992c) found that Douglas-fir (*Pseudotsuga menziesii* [Mirb.] Franco) rooted cuttings survived as well as seedlings and transplants when matched for genetics, stem caliper and root system quality. International Forest Seed Company (Tom Caldwell, pers. comm.) has also found that rooted cuttings of loblolly pine (*Pinus taeda* L.) have higher survival than seedlings of comparable genetic quality. Cuttings of radiata pine have been found to exhibit better survival than seedlings in a harsh Mediterranean climate (Ritchie et al. 1992a).

All of the reasons for the better survival of cuttings are not well understood. Douglas-fir rooted cuttings tended to be more cold-hardy and had a deeper dormancy than did seedlings in one study (Ritchie et al. 1992d). In some cases the cuttings also had larger root collar diameter than seedlings which may have improved their survival. Libby and Rauter (1984) point out that seed from open-pollinated seed lots from orchards may have a greater number of dominant lethal alleles due to natural self-pollination, whereas select trees which are vegetatively propagated should have few to none depending on the age of selection. Competitive dominance may result in higher mortality in very small trees found in seed-derived populations as mentioned above. In some cases, rooted cuttings may have greater root biomass than seedlings.

While rooted cuttings cannot always be counted on to have better survival than seedlings, neither should one assume that cuttings will have poorer root systems or poorer adaptation to field conditions.

Other Advantages

The benefits mentioned above are the primary ones cited by organizations involved in applied vegetative propagation programs. Other more subtle but important advantages, especially for clonal forestry, are discussed in detail by Libby and Rauter (1984). They include capturing gains due to

genotype-environment interaction (matching clones to site), avoidance of pollen contamination, ease of hybrid production, and ease of eliminating and adding clones to hedge orchards versus seed orchards. Another advantage is that vegetative propagation provides a method of producing valuable species such as Alaskan yellow cedar (*Chamaecyparis nootkatensis* D. Don.) which cannot be propagated easily from seed (Ritchie et al. 1992a).

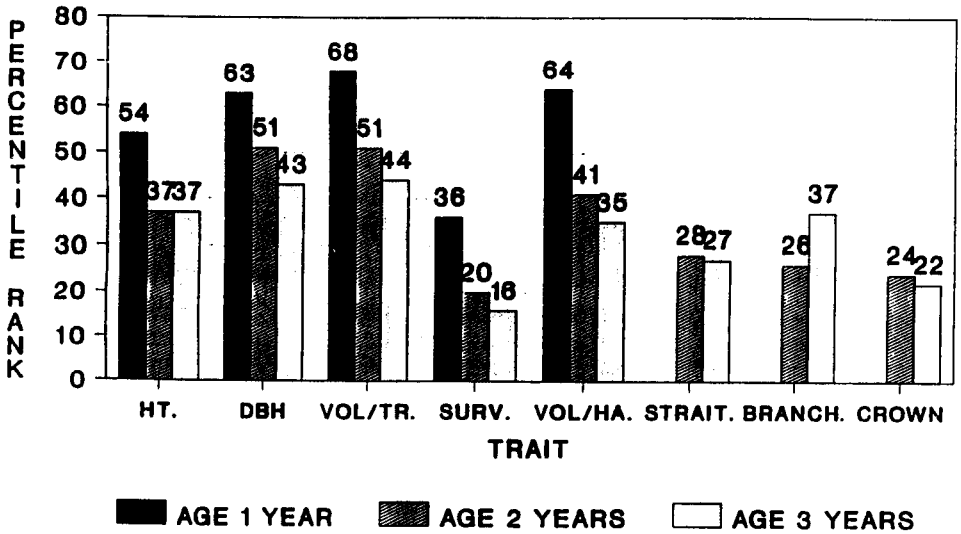

Figure 1. Average percentile ranking of seedling check lots versus clones of Eucalyptus grandis in 16 trials in Colombia

(From Lambeth et al. 1992)

TREE IMPROVEMENT STRATEGIES AND PROPAGATION SYSTEMS

As already discussed, there are a number of reasons for using vegetative versus sexual propagation. There are a number of strategies for vegetatively multiplying a select tree or clone as well. Figures 1 and 2 illustrate several of the commonly used propagation systems in applied programs as well as some systems that are at the research stage. Some of these options (referred to as "clonal options") produce clones as the final genotypes (Figure 1) while others (referred to as "seed-vegetative propagation options") simply multiply desirable seed lots (Figure 2). The appropriate system depends greatly on the ease of rejuvenation (or preservation of juvenility) of a select tree, and rooting environment requirements. Examples will be used to describe the options in Figures 1 and 2 where possible. In some cases, both rooting and nursery facilities may not be necessary but both are shown because it is the most common system for vegetative propagation. "Propagation lab" indicates that a laboratory or other highly controlled and/or aseptic conditions may be required, especially for tissue culture and embryogenesis.

Clonal Options

Clonal options are usually preferred because they provide greater genetic gain and produce a uniform crop for commercial use. These options entail the vegetative multiplication of individual trees to commercial quantities, and there are a number of approaches being used or at least attempted.

Option 1.--In this option, cuttings are taken directly from trees and planted directly into a commercial plantation. This is the approach of James River Corporation in the floodplains of the Colombia River in Oregon and Washington. Cuttings are taken from second and third order branches from the best clones in plantations (Table 1). This, the simplest and cheapest of options, works when the species roots well under field conditions

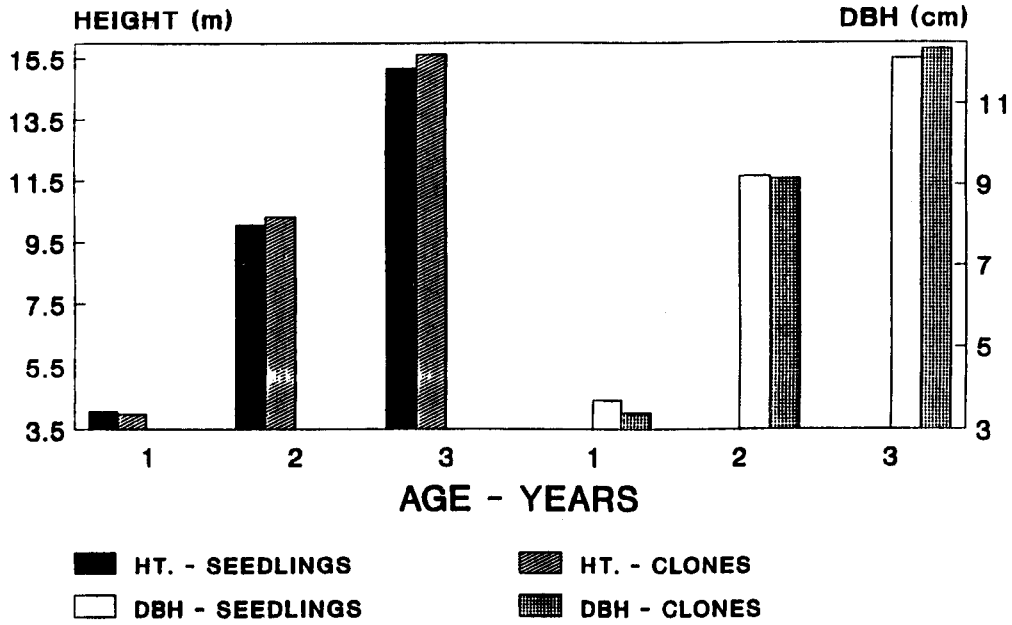

Figure 2. Mean heights and diameters of seedling check lots versus clones of Eucalyptus grandis in 16 trials in Colombia

(From Lambeth et al. 1992)

and when tree branches are sufficiently "juvenile" for good growth after rooting. Most species require more elaborate nursery conditions for rooting or a cutting orchard for rejuvenation or maintenance of juvenility using sprouts from stumps or hedges.

Option 2.--In Japan, branches are collected from "mature" trees and either rooted directly in the nursery and, from there, go directly to a plantation, or they are rooted for purposes of establishing a cutting orchard from which commercial levels of cuttings are collected. This option is more akin to the system used for eucalypts with the exception that the operation begins with branches from a mature tree, an option which is unworkable in eucalypts due to maturation.

Option 3.--This system is used throughout the tropics for eucalypt species. After identifying the select tree it is necessary to produce stump (or basal) sprouts which are more "juvenile" than the branches of a mature tree. Branches of large trees typically do not root well and have mature morphological characteristics shortly after rooting. Another difference between this option and options 1 and 2 is that it is usually necessary to have a special (high humidity) facility for rooting as well as an outdoor nursery area for acclimatization.

Option 4.--For most species, it is necessary to somehow rejuvenate or maintain juvenility of a select tree for ease of propagation and desirable growth. It appears to be easier to maintain juvenility than to rejuvenate. Many programs are aimed at maintaining juvenility of a genotype which has been cloned for purposes of testing under field conditions.

One promising avenue now under intensive research involves the clonal multiplication of individuals through somatic embryogenesis. For a genotype, some somatic embryos (or sprouted tissue) can be placed in cryogenic storage while others are used in the establishment of clonal trials. Once the clone has been tested, the stored material is then retrieved and again multiplied in a propagation lab and further propagated in a rooting facility and/or nursery setting. Commercially, the simplest option would be to further multiply stored embryos to commercial level, encapsulate them and sow them directly into a nursery much as is done with seed. However, a great deal more research is needed to make artificial seed a viable option. Furthermore, it remains to be seen whether laboratory cultures can retain their juvenility indefinitely. Do they mature with time, as do trees and hedges?

Option 5--When juvenile material can not be cryogenically stored it may be possible to maintain clones in hedges while the clonal test is being conducted. This is the approach used in clonal programs of Norway spruce and was being used by International Forest Seed Company for loblolly pine (Foster and Shaw 1987). The difficulties in this option are the cost of maintaining hedges for all clones tested and the fact that it may not be possible to maintain juvenility long enough. Hedges typically mature to some extent when compared to seed-derived plants (Bolstad and Libby 1982). In some cases, a certain degree of maturation can be tolerated, i.e., the effects of maturation are offset by the high genetic value of the clones produced.

Options 6 and 7.--For several years, it was thought that propagules from callus tissue or embryogenesis of mature tissue from a select tree would result in rejuvenation because they would be formed from undifferentiated tissue. However, embryogenesis of mature tissue has proven to be quite difficult and tissue culture plants from mature tissue tend to exhibit mature characteristics (Greenwood et al. 1991). Although this was the most common approach in the beginning of this area of research, it is receiving less and less emphasis.

Seed - Vegetative Propagation Options.--When rejuvenation or maintenance of sufficient juvenility is not possible, then other options must be used; however they typically serve only for bulking up high value seed. These options begin with seed or scions from a select tree but depend on the production of seed somewhere in the propagation system. For purposes of demonstration, conventional systems which produce seedlings as well as vegetative propagation options will be shown. The objective is to show that these vegetative propagation systems have competing technologies of similar genetic gain which rely on seed production for commercial plantings.

Option 8.--Seed can be taken directly from a select tree in a forest or genetic test. Such seed may not be found in commercial quantity, in which case it may be desirable to multiply it vegetatively. An example is the approach being used in Colombia for both *Pinus tecunumanii* (Schwerd.) and *P. maximinoi* (H.E. Moore). These are two highly promising species which are only beginning to be used for commercial plantations. The price of unimproved *P. tecunumanii* seed from Central America placed in Colombia is about $500/kilo and, as yet, good seed production sites have not been found in Colombia. As stated earlier, some tropical pines produce little to no seed in areas near the equator. Seed from select mother trees in Central America have been tested in Colombia and Smurfit Carton de Colombia is returning to the original select mother trees and collecting seed which is being multiplied by creating hedges and rooting cuttings. This same approach is even more critical for reducing foxtailing in *P. maximinoi*. This species normally foxtails at very high percentages but studies have shown that the progeny of some mother trees produce very low percentages of foxtailing while others foxtail at 100% (Urrego and Lambeth 1989).

Option 9.--The seed alternative to option 8 is to collect directly from the select tree for commercial purposes. Examples would be seed production areas (where plantations or natural stands are thinned to leave the best trees) or seedling seed orchards (where genetic trials are genetically thinned).

Option 10.--This is the clonal seed orchard favored by many organizations around the world. Open-pollinated, supplementally-pollinated, control-pollinated and hybrid seed can be produced in an orchard setting. Considerable labor may be required for making controlled or supplementally pollinated seed, but it is still an option that is favored in many regions of the world.

Option 11.--In this option, high value (or scarce) seed from the clonal seed orchard is used to establish a cutting orchard (usually hedges) from which sprouts are used for rooted cutting production on a commercial scale. This approach is being used by a number of organizations including the Douglas-fir program at Weyerhaeuser Company (Table 2). Hedges are retained only a short period of time in order to assure the production of juvenile cuttings. This approach is typically used when plants from hedges which are maintained for too many years, lose desirable characteristics such as rootability or growth rate. This approach is also used when there is insufficient evidence to determine maturation effects in long-term hedges.

Option 12.--Again, there is hope that embryos from somatic embryogenesis may be encapsulated and their sowing mechanized in such a way that option 12 may be cheaper than option 11. Somatic embryogenesis in this system would be easier than it

would be in options 6 and 7 since it would be conducted on seed embryos.

COMMERCIAL SCALE APPLICATIONS IN THE UNITED STATES

There are two commercial scale vegetative propagation programs which offer contrasting species (hardwood versus conifer) and different approaches to vegetative propagation (clonal versus bulking up of seed) and age of the programs (32 versus 5 years).

James River Corporation

James River Corporation (Table 1) has planted poplar cuttings in the South (the approach is similar to option 3 in Figure 1) since 1960 and in the Northwest (a system similar to option 1 in Table 1) since 1982. The program is justified primarily by the fact that planting of cuttings directly in the field is considerably cheaper than production of seedlings in a nursery for commercial plantings, and by the substantial gains that can be made in the commercial deployment of superior clonal plantations. Program features can be seen in Table 1.

Tree Improvement Strategies.--The basic tree improvement strategy in the Northwest is interspecific hybridization (eastern X black cottonwood) followed by combined family and within family selection. The same strategy is used in the South though species hybridization is replaced with intraspecific matings of eastern cottonwood (*Populus deltoides* Bartr. ex Marsh.). Selected eastern cottonwood genotypes serve in the breeding programs of both regions, having been chosen on the basis of performance in clonal trials in the South. Currently, parental selection of black cottonwood (*Populus trichocarpa* Torr. and Gray) takes place in natural stands. Future breeding stock will come from a 1993 trial of 1,700 black cottonwood clones. Implicit in this strategy is the assumption that clonal values are suitable indicators of breeding values in both inter- and intraspecific matings. Currently there are 58 and 102 full-sib families of eastern cottonwood and hybrid cottonwood, respectively, in various stages of clonal evaluation.

Factorial mating designs with parental assortative pairings are used in both regions. Progeny are evaluated for one year in a nursery after which time approximately 50% are mass-selected for clonal screening. Initial selections are made at age two in the Northwest and at age four in the South. Clonal multiplication of these selections commences with greenhouse propagation of 1,000 rooted cuttings per clone. The multiplication operation continues another three years through stoolbed culture in the South and through field plantings in the Northwest. At this point, a rotation-age evaluation of the screening trial is conducted to either confirm or refute the superiority of the earlier selections. Replicated monoclonal plots are also established to provide realistic growth and yield data.

In the long term, the southern improvement program will focus on recurrent breeding. Long term breeding in the Northwest will not focus on advanced generations as there does not appear to be much promise in the F_2 hybrid generation. Consequently, a reciprocal recurrent selection program designed to improve both parental species for hybridization is being developed.

On the operational side, both programs will likely increase their acreage under cultivation in future years. With continued clonal development, it is anticipated that yields will increase or rotations will shorten. Of equal concern is the desire to broaden the genetic diversity of the operational clone pools, especially in the Northwest where other hybrids may be pursued.

Coppicing in second rotation stands is the only competing technology to the development of new clones. While refined in the South, questions remain about effective ways to use coppice in the Northwest.

Weyerhaeuser Company

Main program features can be found in Table 2. The program began in 1987 with the objective of bulking up elite full-sib seed of Douglas-fir (option 11). Even if control crossing were possible, orchard capacity would be sufficient to produce only eight percent of regeneration demands. Vegetative propagation easily moves this figure up to 100%.

Only one-year-old stock plants are used for cutting production in order to avoid negative effects on growth and rooting due to maturation. Growth of stock plants is accelerated. By the end of the year, stock plants produce 50 cuttings which are rooted in

the greenhouse and then transplanted to a conventional bareroot nursery (essentially a 1 + 1). The cost of cuttings is two to three times higher than that of seedlings. The target is 1.5 times that of seedlings.

Rooted cuttings are now being planted on the highest site land near the mill in order to get the highest financial return for high value families. In addition, they are not planted on sites which have a low probability of remaining in company ownership through rotation such as suburban growth areas and scenic corridors.

Tree Improvement Strategies.--Select full-sib families for bulking up are identified from about 4,000 parents across six breeding zones in 8- to 12-year-old first generation tests. These selections come either from tested crosses or from elite parents which have not been tested as specific crosses. Second generation seed will be available for testing within one or two years. It is anticipated that the best second generation families will be bulked up with cuttings in a similar manner.

There is a clearly articulated strategy and rules for allocation of families to sites, maximum contiguous acres of a family, maximum percentage contribution of a single family to any year's total regeneration and the number of families to be planted.

The program is expected to grow considerably in the next few years if cost reduction efforts continue to be successful and if field tests continue to demonstrate high gain potential. Ritchie (1992b) provides more detail about the Weyerhaeuser program.

PRINCIPAL PROBLEMS IN APPLIED VEGETATIVE PROPAGATION PROGRAMS

Maturation of Stock Plants

Perhaps the most commonly cited problem which limits the development of applied rooted cutting programs is reduced growth and rooting potential resulting from maturation of stock plants (Armson et al. 1980, Arnold and Gleed 1985, Bolstad and Libby 1982, Gill 1982, Greenwood et al. 1991, Johnsen 1985, Libby 1974, Rauter 1983, Shelbourne 1987, Spencer 1987, Struve and McKeand 1990). Nonetheless, some are exploiting the mature characteristics (Armson et al. 1980, Arnold and Gleed 1985, Bolstad and Libby 1982, Gill 1982, Libby 1974, Libby and Rauter 1984, Spencer 1987), especially those programs aimed at high quality, solid wood products. However, many programs either do not rely on solid wood products or do not find that the improved quality of more mature cuttings is sufficient to offset the losses in yield or increased nursery costs due to poorer rooting. Maturation is a problem in many species, perhaps even in stump sprouts of eucalypts (Lambeth et al. 1992) which are commonly rooted on a commercial scale and are thought to be without maturation problems.

One difficulty that tree breeders may have in the assessment of rooted cuttings is evaluation time. Clones from plus trees of *Eucalyptus grandis* had more mature characteristics than did nonselect seedlings with less branching, larger leaves and slower growth in the first year (Lambeth et al. 1992). By the second year (1/3 rotation) the clones had surpassed seedlings in growth and their superiority increased through the third year (1/2 rotation) (Figures 3 and 4). This delayed expression of superiority of clones over nonselect seed lots has also been observed in clonal programs in Brazil (Dr. Bruce Zobel, pers. comm.) and South Africa (Dr. Neville Denison, pers. comm.). It is suspected that the clones are more mature than seedlings and, therefore, have slower relative growth rate in the early establishment year(s). However, as seedlings pass out of the exponential growth phase associated with juvenile stock, the clones begin to express their true genetic superiority in the mature phase of growth.

It will become more important to understand the growth curve of rooted cuttings (especially those from older hedges) and their state of maturation versus seedlings as operational programs develop. This will be especially important for long-rotation conifers. The evaluation period for comparison seedlings versus cuttings may depend on a difference in shape of the growth curve as has been observed in eucalypts. In the compressed time frame for eucalypts, clones surpassed seedling checks between 1/3 and 1/2 rotation age. If the same occurs in conifers then many trials may be too young to evaluate the difference between cuttings versus seedlings for the long term. On the other hand, if cuttings are considerably older physiologically than seedling counterparts, they may never catch up in growth rate.

Cost

In most conifer programs, cost of cuttings is still considerably higher than that of seedlings primarily because of the special conditions needed for rooting and the labor-intensive nature of rooted cuttings (Arnold and Gleed 1985, Gill 1982, Greenwood et al., Ritchie 1992b). Three things will make the costs of vegetative propagation systems more favorable: 1) Mechanization of harvest and sticking of cuttings or transplanting of rooted cuttings will lower cost considerably. Mechanization has not received much emphasis in the past because of the uncertainty of the applicability of rooted cutting systems. 2) Simpler rooting systems will be developed and costs of facilities will drop. 3) System costs will be examined more closely and, in many cases, it will be concluded that a rooted cutting system will be competitive with seedlings, especially when larger genetic gains are possible with cuttings. In some programs, seed orchards will be obsolete or at least much smaller orchards will be established with the sole objective of feeding rooted cutting programs.

The system advantage of rooted cuttings will not only be in the form of cost savings, but also through faster implementation of genetic gains (Arnold 1985, Gill 1982, Matheson and Lindgren 1985, Libby 1974, Libby and Rauter 1974, Rauter 1983, Ritchie 1992b).

Environmental Restrictions

Given the current worldwide concern about biodiversity and monocultures, many researchers and plant propagators are concerned that vegetative propagules may be regulated at some future date to the point that full potential gains may not be exploited. Furthermore, it is possible that investments in biotechnology and vegetative propagation may be lost if the planting of genetically engineered genotypes or clones is too restrictive. Such concerns may discourage the development of vegetative propagation programs. For example, the Swedish government places restrictions on how clones should be tested and specifies the number of clones that must be planted in mixtures (Anon. 1982). In Sweden, the potential benefits of uniform clonal plantings can not be realized because monoclonal plantings are illegal.

It would behoove forest geneticists to begin studies on the genetic variation and risks of different deployment strategies of not only clones but seed orchard families as well whether they be open-pollinated or full-sib families. For example, it has been suggested that genetic diversity of clonal mixtures may be greater than that of open-pollinated families from seed orchards when appropriate mixtures are used (Libby and Rauter 1984). What about a mosaic of monoclonal plantings versus clonal mixtures or open-pollinated family blocks versus plantations of unimproved seed? From a legal standpoint, what are the private landowner's rights in deciding which genotypes to plant and in deciding how to deploy them? These issues must be dealt with quickly in order to avoid problems that may arise when significant quantities of plant material emerge from biotechnology and vegetative propagation programs.

Competing Technologies

Those vegetative propagation options which bulk up high value seed lots such as full-sib families (Figure 2) must compete with seed orchard options which, perhaps, have not been investigated thoroughly enough. These include supplemental mass pollination, control crossing, two-clone orchards and relocation of orchards to other areas of faster and more abundant production of cones, higher seed set, no pollen contamination and low labor costs for making crosses. Slash pine (*Pinus elliottii* Engelm.) is a good example of the latter. In its natural range, slash pine produces commercial quantities of seed in orchards at 10 years of age or later and suffers from considerable pollen contamination. In Zimbabwe or southern Brazil, seed production occurs at five years of age, is more abundant than in the U. S., areas of low pollen contamination can be found, and labor costs for making controlled crosses is lower than in the U.S.. Scion export for seed orchard establishment overseas and seed import costs would be nominal but import and quarantine restrictions would have to be studied very carefully.

Tree breeders must take some risk in deciding whether or not to put research into vegetative propagation or seek alternative seed production systems for the production of full-sib families. This decision is difficult when there is insufficient research in either area. Furthermore, somatic embryogenesis is a competing technology for rooted cuttings and seed orchard crosses in the production of full-sib families since all three techniques would produce the same level of genetic gain, assuming one system has no more maturation problem than the others.

CONCLUSIONS

Worldwide commercial applications of vegetative propagation are increasing at a dramatic rate. Exploited advantages are increased growth rate from high value genotypes in the form of clones or bulked up full-sib families, increased crop uniformity and predictability of wood quality and other characteristics, greater quality of final products associated with more mature characters such as reduced branching and knot size, faster multiplication of high value families due to slow seed orchard production, and reduced system cost by elimination or downsizing of seed orchards. There are several genetic strategies and propagation systems in use. The appropriate strategy depends on the ease of propagation and rejuvenation (or maintenance of juvenility) and potential genetic gains. Some produce clones for commercial planting while others simply multiply high value seed lots. The cost of the latter must be competitive with alternative breeding technologies such as supplemental pollination, two-clone orchards or control crossing on a commercial scale.

Primary limitations in the implementation of vegetative propagation and reforestation are cost due to the labor-intensive nature of rooted cuttings or tissue culture, maturation of stock plants (usually hedges) which reduces rooting percent and growth rate in the field, and the fear of environmental restriction on the use of new materials from expensive research programs. Some of these limitations are being overcome by research into cost reduction methods through mechanization and studies of methods to maintain juvenility (or rejuvenation) of select trees or clones.

The United States lags behind other countries such as Brazil, The Congo, Japan, South Africa and several western European countries in the level of use of vegetative propagation on a commercial scale. Two notable exceptions are the programs at James River Corporation, which plants clonal plantations of poplars on floodplains of the Mississippi and Columbia rivers, and Weyerhaeuser Company, which has a rapidly growing program of vegetative multiplication of high value full-sib families for regeneration in Oregon and Washington.

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Table 1.--Features of a James River Corporation rooting cutting program

SPECIES:	South - Eastern Cottonwood (*Populus deltoides*) Northwest - Black Cottonwood (*Populus trichocarpa*) X Eastern Cottonwood Hybrid
REGION:	Mississippi (Mississippi and Louisiana) and Colombia (Oregon and Washington) Rivers Floodplains
OBJECTIVE:	Produce Fast-Growing Clonal Plantations
REASON:	Best Clones From Full-Sib Families Exceed the Family Mean by 10% in Height and 20% in DBH

REGENERATION ACTIVITY:

SOUTH
- Since 1960 - 7,000 ha Planted
- Clones 67% of 690 ha Annual Planting

NORTHWEST
- Since 1982 - 4,500 ha Total
- Clones 100% of 400 ha Annual Planting

FINAL PRODUCT:

SOUTH
- High Quality Coated papers

NORTHWEST
- Groundwood Communications Grade Paper

ROTATION:

SOUTH
- 10 Years

NORTHWEST
- 6 Years

PROPAGATION SYSTEM:

SOUTH
- Cuttings from 1-year-old Whips in Stool Beds
- 4-Year Rotation in Stool Beds
- Cuttings 20-50 cm Long and .6-2.5 cm Diameter

NORTHWEST
- Cuttings from 1st and 2nd Order Branches in 2-year-old Commercial Stands
- Cuttings 30-35 cm Long and .6-2.0 cm Diameter

BOTH REGIONS
- Rooting Directly in the Field (95-100% Success)

LIMITATIONS AND CONCERNS: Genetic Diversity in Breeding Programs

FUTURE ACTIVITY:

SOUTH
- Recurrent Breeding and Testing

NORTHWEST
- Reciprocal Recurrent Selection for Good Hybrid Performance

BOTH REGIONS:
- Emphasis on Better Clones from Breeding Programs
- Increased Clonal Planting

Table 2.--Features of a Weyerhaeuser Company rooted cutting program.

SPECIES: Coastal Douglas-Fir (*Pseudotsuga menziesii*)

REGION: Western Washington and Oregon

SITE TYPES: High Site Index, Low Elevation

OBJECTIVE: Multiply Limited Quantity of High Value Orchard Seed such as Full-Sib Families

REASON: 15-25% Gains in Site Index

REGENERATION ACTIVITY:

1987 - First Crop
1991 - 35 ha
1992 - 40 ha
1993 - 700 ha Planned
1994 - 800 ha Planned
Of 10,000 ha Total Annual Regeneration

FINAL PRODUCT: High Value Solid-Wood Products, Pulp

ROTATION: 40-50 Years

PROPAGATION SYSTEM:

- 1-Year-Old Potted Stock Plants in a Greenhouse
- Winter Dormant Cuttings - approx. 50 per Stock Plant
- Greenhouse Rooting
- Transplant to a Bareroot Nursery in the Fall
- One Additional Year in the Nursery

GUIDELINES:

- Limited Contiguous Area of a Single Full-Sib Family
- Limited % of Area of Annual Regeneration to a Single Full-Sib Family

LIMITATIONS AND CONCERNS:

- Cost (Target 1.5 x Seedlings)
- Future Environmental Restrictions on Deployment of Select Material

FUTURE ACTIVITY:

- Increased % Regeneration with Rooted Cuttings
- Perhaps Smaller Seed Orchards Which Will Produce Control Cross Seed for Vegetative Propagation

RESEARCH APPLICATIONS WITH VEGETATIVE PROPAGULES[1]

Darroll D. Skilling, Kenneth F. Raffa, Daniel J. Robison, and Paul Berrang[2]

Abstract.--Vegetative propagules have recently received more attention in forest tree improvement due to more efficient techniques for plant multiplication including tissue culture. Vegetative propagules are being used in research on air pollution, entomology, and pathology to develop more sensitive tests for screening putative resistant materials. These techniques offer both speed, and the ability to produce large numbers of new genotypes.

INTRODUCTION

The use of vegetative propagules probably dates back prior to written history when the first humanoid poked a willow branch in a sandbar and came back later to see a young tree growing in place. So what we are discussing today is not exactly the cutting edge of science. What has changed, however, is the ability to take ancient techniques and modify them to provide us with tools that can be used to help us in today's tree improvement work. In this paper we discuss the value of vegetation propagules in research. We describe some systems that have important research applications. Although these research techniques may help us develop trees with genetically improved traits, they are just another approach to tree improvement. Information from these techniques will supplement more classical breeding programs, but they will not replace them.

The plant material developed in the research laboratory must still be subjected to field testing and may become stock material for future tree breeding. Having made this qualifier, let's look at some of these techniques and see how vegetative propagules can be used to study interactions between insects, air pollution, and diseases as stress components in forest trees.

In research, we try to hold variables constant except for the independent variable we are studying. With forest trees, this can often be difficult as each individual tree is a variable and in most cases is somewhat different genetically from every other tree. To study uniform populations we devise systems to combine members of various families so that each treatment has approximately the same representative genotypes present. Through statistical methods it is then possible to separate out the variation due to treatment from that variation due to genetic variability in the plant material. A more sensitive test, however, is to deal with identical genotypes in each treatment. This can be achieved by the use of vegetative propagation systems in research studies.

In forest pathology we have a system called the "disease triangle". In this triangle the development of a forest disease is the result of three variables. These are the genetics of the host, the genetics of the pathogen, and the variation within the environment. A change in any one or more of these three parts of

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the triangle may have a major impact on disease development.

If we want to study the genetics of the fungus we must hold the genetics of the host and the environmental factors constant and vary only the races of the pathogen. If we wish to study the genetics of the host, in this case a tree, we maintain the genetics of the pathogen constant as well as the environmental factors and vary only the genetics of the tree. This is why pathologists working with identical vegetative propagules in tissue culture can detect smaller differences in genetic resistance than would be possible in field studies. How important these small differences are depends on the purpose of the study.

The idea of working with a single tree genotype under controlled environmental conditions is of course not limited to forest disease research. In this paper, we will discuss insects, air pollution, and diseases, but the general principal will remain the same.

APPLICATIONS TO AIR POLLUTION RESEARCH

General Comments

Traditionally, air pollution research has been done by plant pathologists and, if one accepts some pathologists' definition of disease, air pollutants are just a type of disease. In air pollution, however, the disease triangle is severely truncated because pathogen genetics is not a factor. Within the forest air pollution field, the triangle is often even more reduced because most researchers have only a passing interest in the genetics of the host. The reasons air pollution researchers would use vegetative propagules are similar to the reasons most forest pathologists would use them. First, the use of genetically identical plants makes it possible to obtain a more uniform plant response to specific types of air pollution stress. Second, the ability to repeatedly replicate a specific genotype means that vegetative propagules can be used to increase the continuity between experiments or over space and time. Third, the ability to produce multiple copies of individuals makes it possible to investigate aspects of genetic variation that are not available with plant material grown from seed. In many cases, researchers obtain several of these benefits simultaneously.

Air pollution researchers in agronomy and horticulture have made extensive use of inbred lines and hybrid varieties in their work. They have made tremendous advances in their research due, in part, to their ability to quickly and economically produce large numbers of genetically similar plants. Research on ozone injury on tobacco is just one example of their successes. Starting in the early 1950's tobacco growers throughout eastern United States began reporting crop injury that seemed to be related to the weather. It was not until the late 1950s that ozone was recognized as the cause of this injury. By 1960 tobacco growers were starting to plant resistant varieties (Heggestad 1966) and today ozone injury is rare on this crop. Breeders of a wide variety of crops continually make improvements in the air pollution tolerance of a wide variety of species. Inbred lines and hybrid varieties like those used in agronomy and horticulture are practically non-existent in forestry, but clones produced through vegetative propagation have the potential to provide forest air pollution researchers with many of the same benefits. However, to date, there have been very few applications of this technology to forest air pollution research.

When forestry research lags behind agricultural or horticultural research, it is often due to the fact that foresters enter the field at a later date. That is not the case at least with ozone. Researchers working with trees were some of the first people to study the effects of ozone on plants.

Examples of Work

Verification of Injury Observed in the Field.-- Some of the earliest ozone work done with forest trees involved the use of vegetative propagation. In the late 1950's the USDA Forest Service began to investigate a decline of eastern white pine in the southern Appalachians that was called post emergence chronic tipburn. There was a great deal of apparent variation in susceptibility to this disease with some individuals showing extensive damage while others showed no damage. Early work indicated that the disease was not associated with pathogenic fungi (Berry and Hepting 1964) or nutrition (Dochinger 1964). Reciprocal grafting experiments established that the disease was not graft-transmissible and that differences in susceptibility between seedlings seemed to be genetically controlled (Berry and Hepting 1964). In the early 1960s, Berry and Ripperton (1963) established ozone as the probable cause of this

disease by producing clones of susceptible individuals and showing that ramets maintained in ozone-free air did not develop the disease while plants fumigated with ozone did develop the disease.

One might expect that the success of these early researchers might encourage later forest air pollution workers to make frequent use of vegetative propagation to minimize genetic variation in susceptibility. However, this has not been the case. Traditionally, foresters have used genetically diverse groups of trees propagated from seed to reforest areas, and most forest air pollution workers have chosen to work with tree seedlings.

Develop Better Bioindicators of Ozone.--Pioneering ozone researchers developed clones of plants that were useful as indicators of the presence of phytotoxic levels of ozone (Berry 1973). Interest in this work declined as accurate mechanical monitors became more widespread. Interest in this technique has been revived recently because it is viewed as a way of monitoring the effects of ozone rather than its mere presence.

The first bioindicators consisted of a single clone or inbred line which was selected for sensitivity to the pollutant of interest. Many factors other than ozone can affect the response of these plants to ozone including soil moisture, nutrient availability, and temperature. Heagle (1991) has developed a pair of white clover (*Trifolium repens* L.) clones, one sensitive to ozone and one resistant, for use as bioindicators. When the growth of just one of these clover clones is compared to ambient ozone concentration, the correlation is, at best, weak. However, these two clones were selected from the same clover cultivar and presumably react similarly in response to most other environmental factors other than ozone. When the relative growth rates of these two clones were compared in ambient ozone there was a strong correlation (Heagle, USDA Agricultural Research Service, Raleigh, NC, personal communication).

The USDA Forest Service has the responsibility of preventing air pollutants from affecting resources in Class 1 Wilderness Areas. The agency is also prohibited from installing conventional mechanical monitors in these areas by wilderness legislation. One option being used is collecting data on symptoms of injury on sensitive plants in the field. Field surveys indicate that phytotoxic levels of ozone exist in many wilderness areas, but natural variation in ozone tolerance within species limits the conclusions that can be drawn from data collected in natural populations. The Center for Forest Environmental Studies plans to develop paired clones of native bioindicators that would function much like the clover clones already described.

Study Patterns of Genetic Variation.--Relatively little work has been done with the genetics of air pollution tolerance in trees, but there tends to be a substantial amount of variation for these traits within species of forest trees. For example, Karnosky and Steiner (1981) reported that some green ash (*Fraxinus pennsylvanica* Marsh.) provenances showed 10 times as much sulfur dioxide injury and 60 times as much ozone injury as other provenances of the same species. They also found significant differences among families within provenances, but like most of the researchers who have studied genetic variation in air pollution tolerance, they were unable to evaluate differences among individuals within families because they were working with seedlings.

In the few cases where researchers have vegetatively propagated individuals within clones they have found that differences among individuals can be a major component of variation. While studying the effect of sulfur dioxide on height growth of Norway spruce (*Picea abies* (L.) Karst.), Scholze and Venne (1989) found that in clean air, differences among provenances accounted for most (54-63%) of the genetic variation, but that in the presence of the pollutant differences among clones within families accounted for most (30-32%) of the variation. Variation due to differences among families was relatively small regardless of sulfur dioxide level. Berrang and Karnosky (1986, 1991) did not evaluate differences among families within provenances, but they did find that differences in ozone tolerance among clones within provenances of quaking aspen (*Populus tremuloides* Michx.) were substantially larger than differences among provenances.

If the amount of variation among individuals in these two species is typical of other trees, geneticists working with air pollution tolerance would have considerably more variation to work with if they included vegetatively propagated clones in their studies.

Another advantage of using clonal propagation to study patterns of genetic variation is that the actual

plants in the population are being sampled rather than their offspring. When one propagates population samples from seed, the samples can include seedlings that are extremely sensitive to air pollution and would not survive long in ambient air. Berrang et al. (1986, 1991) showed, using vegetatively propagated aspen, that populations from polluted areas do not include the sensitive clones found in pristine areas suggesting that sensitive individuals are eliminated by ambient levels of air pollution in polluted areas.

Breeding for Air Pollution Tolerance.--In reference to this topic, Zobel and Talbert (1984) stated "there is no area of forest tree breeding in which greater emphasis is needed." However, major programs specifically designed to develop air pollution resistant trees do not exist in the United States. This is probably due, in part, to the fact that the most common pollutants in this country are regional. Because the effects are spread uniformly over broad areas, it is difficult to convincingly demonstrate that the problem is severe enough to justify a major breeding program. Forest air pollution researchers have instead concentrated on determining the magnitude of air pollution effects.

There are only a few estimates of narrow sense heritability of air pollution tolerance in trees but they suggest heritability is low to moderate for height growth (Gerhold 1977, Scholz and Venne 1989). Given the fact that there are tremendous differences in tolerance to air pollutants among individuals, specific combining ability and/or non-additive components of variation may be important components of genetic control. If this turns out to be the case, the ability to vegetatively propagate large numbers of ramets from selected individuals should be an important component of breeding programs.

Study Mechanisms of Resistance.--There is a growing interest in discovering the various mechanisms by which trees and other plants can resist the effects of air pollutants. Some of these mechanisms allow plants to avoid exposure to air pollutants while others enable plants to avoid reductions in growth when they are exposed to air pollutants.

Air pollutants must enter the plant leaf for exposure to occur. Stomates are the main barrier to the uptake of gases, and several agricultural crops appear to avoid air pollution exposure by closing their stomates. There is a possibility that those characteristics that affect the transport of pollutants within the leaf may affect ozone sensitivity. Examples of such characteristics could include the amount of intercellular space in the leaf mesophyll and the reactivity or solubility of pollutants with cell membranes. Little evidence exists to show that these characteristics are related to air pollution tolerance in trees.

Some plants may tolerate exposure to pollutants by scavenging ozone or the free radicals formed from ozone. One example of such a mechanism would be actively increasing levels of ascorbic acid in response to exposure to ozone. This is true in several agronomic crops and Chevone et al. (1989) reported greater concentrations of ascorbate in ozone-resistant genotypes of white pine than in ozone-sensitive genotypes of the same species. Very little work has been done with trees in this area, but extensive work with other plants suggests that several enzyme systems may play a role in protecting plants from the effects of air pollutants including superoxide dismutase (Bennett et al. 1984) and peroxidase (Jager et al. 1985).

There is limited evidence that suggests plants may tolerate exposure to pollutants by developing repair mechanisms. There is very little evidence of this sort for trees, but work with agricultural crops has shown that exposure to ozone can increase concentrations of ATP (Pell 1974) or that proportion of respiration presumably required for maintenance (Amthor and Cumming 1987).

Mechanisms that depend on stomatal closure would presumably require that intact plants be used when genotypes are screened for tolerance to pollutants. However, it may be possible to develop in vitro tests for screening genotypes in cases where mechanisms depend on scavenging ozone or repair of injury. Tree breeders may be able to use somaclonal variation to obtain more resistant genotypes in these cases.

There can be differences among resistant individuals within a single population of a single species in the mechanisms by which they avoid the effects of air pollutants. For example, Karnosky (Michigan Technological University, Houghton, MI, personal communication) has found that some clones from a single population of quaking aspen in Wisconsin avoid injury by closing stomates during ozone exposures. Because there can be such variety between individuals, it may be difficult for

researchers working with differences between families or between provenances to identify physiological mechanisms that explain the differences in sensitivity they have absorbed. Working with single clones would probably increase the likelihood that these researchers could identify specific mechanisms by which plants resist the effects of air pollutants.

Comparing Response of Seedlings and Mature Trees.--Much of the forest tree work has concentrated on seedlings, and an important issue is how closely seedling responses represent the responses of older trees. Two approaches have been used to evaluate differences. One technique involves exposing individual branches of mature trees to air pollutants at the same site that whole seedlings are being fumigated. A second technique assumes some degree of cyclophysis or persistent age effects and involves comparing the responses of clonal material grafted from mature trees with seedlings grown from seed collected on the same trees. Using this technique, Sue Kossuth showed that the response of tissues of different ages to ozone was similar for diameter growth, but not for height growth (SCFRC 1990).

APPLICATIONS OF VEGETATIVE PROPAGATION TO BASIC AND APPLIED RESEARCH ON INSECT RESISTANCE IN TREES

INTRODUCTION

The current interest in tree defense mechanisms arises from both basic ecological and applied forestry perspectives. Basic ecologists are concerned with the selective pressures exerted on plants by insects, and conversely, on insect counter adaptations for overcoming plant resistance traits. Trees are of particular interest because they raise the evolutionary paradox of long-lived hosts co-existing with rapidly reassorting parasites. The forestry interest arises out of the need for improved trees. Tree breeders attempt to identify, select, and breed for heritable traits, including those that may improve overall yield by conferring protection against pests. Both approaches have produced major advances during the last two decades. The ecological perspective has fostered synthesis and development of robust theories. For example, the levels and types of plant commitment to defense can be at least partially predicted based on the relative availability of carbon and nitrogen, insect feeding guild, fitness values of various plant tissues, and plant life histories (Bryant et al. 1985, Coley et al. 1985, Bazzaz et al. 1987, Raffa and Berryman et al. 1987, Mattson et al. 1988, Price et al. 1989). Resistance breeders, on the other hand, have contributed most of our genetic knowledge on plant defense mechanisms (e.g., Hanover 1975, 1980). A number of agricultural, and a few tree, varieties have been successfully developed for insect, or combined insect and disease, resistance.

Progress toward both objectives has been enhanced by the recent integration of these previously isolated perspectives. Basic evolutionary and ecological theories can sometimes provide guidance to breeding and pest management programs. Conversely, genetically well-defined selections and/or hybrids can greatly facilitate analysis and understanding of basic evolutionary processes.

Despite this progress, both approaches have major limitations. Many ecological theories remain largely untested, and are difficult to approach by traditional approaches. Rigorous hypothesis testing requires experimental control of environmental and genetic factors. Natural ecosystems are often highly variable and complex, and while they foster comparative studies, are less amenable to experimentally controlled analysis. Likewise, resistance breeding is operationally very difficult with trees, and has advanced less rapidly than in agriculture. The long generation times and large size of trees can seriously impede the breeding and rapid evaluation needed for improvement programs.

The difficulties facing both basic ecology and applied breeding can be partially overcome by using research models that employ vegetatively propagated materials. Sexually propagated crosses are required to characterize the extent and potential of heritable variation in resistance, but subsequent use of vegetative clones offers the uniform genetic base and control needed to address more specific questions. Full integration of propagation by seed and vegetative means provides the highest likelihood of success (Libby 1973, Dickman and Stuart 1983, Mordiek 1983). Vegetative propagation can also help breeders reduce some of the delays in tree evaluation (Libby 1973). Site-specific requirements, effects of silvicultural treatments, and site-by-treatment interactions can be rapidly and reliably quantified by planting clones in split-plot

designs. Vegetative propagation becomes particularly valuable in contending with the broad mosaic of traits that are required of plantation trees, because these vegetative clones allow for multiple traits to be more rapidly assayed and deployed.

Rapidly Growing Hardwoods as a Model System

Fast growing hardwood species, such as *Populus* and *Salix*, provide an example of trees that are amenable to several methods of vegetative propagation. They also contain a range of heritable traits needed both for testing ecological theories (Price et al. 1989, Whitham 1989) and improving production systems (Dickman and Stuart 1983, Mohrdiek 1983). Within-clone variation is low, which allows environmental effects to be more accurately measured. The high between-clone variation provides a broad genetic background for testing ecological theories and guiding tree improvement programs. Cuttings from rapidly growing hardwoods, for example, can be rapidly produced for glasshouse studies, and thereby expedite research assay and design. Glasshouse and growth chamber experiments must always be verified under more realistic conditions, however (e.g., Robison and Raffa 1992 a,b).

Our work has concentrated on rapidly growing hybrid poplars. The extent to which the potential of these trees will be realized, both for traditional fiber uses and as a biomass alternative to fossil fuels, depends on inter-related biological and economic considerations. The major insect defoliator of poplars is currently the cottonwood leaf beetle (*Chrysomela scripta* F.); two lepidopterans, the forest tent caterpillar (*Malacosoma disstria* Hubner) and the gypsy moth (*Lymantria dispar* L.), also pose significant threats (Caldbeck et al. 1978, Dickman and Stuart 1980, Ostry et al. 1989). Our research team includes specialists with both traditional and molecular expertise. In the following sections, we will not attempt to exhaustively list all of the benefits of vegetative propagules, as a number of excellent reviews are already available (Libby 1974, Toda 1974, Hartney 1980, Copes 1981, Mott 1981, Zobel 1981, Burdon 1982). Rather, we will provide a few illustrations of how these models facilitate both basic ecological understanding and resistance breeding in insect research.

Contributions of Vegetative Propagation Research to a General Understanding of Tree-Insect Interactions

When ecologists evaluate plant reproductive "strategies", there is an implicit assumption that photosynthate allocation to one process, such as growth, is at the expense of other processes such as defense (Lorio 1986, Bazzaz et al. 1987). This view has intuitive appeal, but is difficult to test because of the operational problems described above. These physiological alternatives, if valid, have implications both for evolutionary theory and tree improvement strategies. Vegetatively propagated *Populus* has enabled us to test some of these assumptions (Robison and Raffa 1992 a,b,c). Among 15 hybrid poplar clones assayed for susceptibility to Lepidoptera, Coleoptera, rodents, and *Septoria* fungi, there were no significant relationships between primary productivity and susceptibility to any pest. At least within *Populus*, these artificially developed lines do not support the assumption of "tradeoffs" between growth and defense (Robison and Raffa 1992 a,b,c). Each system is likely to differ, but these results indicate that the assumption of tradeoffs should be rigorously tested despite its intuitive logic. This research also lends caution to the widely accepted and appealing view that plant defense theory can provide strong guidance to breeding programs (Robison and Raffa 1992b). From a positive standpoint, however, this research also suggests that clones having both rapid growth and high resistance properties can be developed.

A second area of interest concerns the relationship between insect behavior and plant defense. Behavioral aversion has long been recognized as a key element of plant resistance to insects (Painter 1941). From a basic evolutionary perspective, behavioral aversion also raises the question of whether some plants have evolved chemicals that innately repel insects, or conversely, whether insects have evolved the ability to orient away from harmful chemicals (Miller and Strickler 1984). The above clones were tested for a variety of forest tent caterpillar performance factors (Robison and Raffa 1992). Early instar larvae preferred clones that best supported larval growth, which lends support to the view that insects select the most beneficial plants. However, there were no relationships between insect choice, either by larvae or adults, and larval survival, and late instar larvae sometimes preferred clones that delayed their development times. These results do not support the hypothesis that insect preferences always reflect optimal choices. This example illustrates how vegetatively propagated clonal *Populus* can provide the strictly controlled experimental conditions

needed to dissect specific components from complex plant-insect interactions, and thereby refine overly generalized hypotheses. It also adds to our understanding of the multifaceted components of plant defense systems, even against a single herbivore.

A third area of plant-insect interaction theory concerns differences between specialists and generalists (Bernays and Graham 1988). This issue also impacts applied forest entomologists and silviculturalists, because the feasibility of employing multiple-species plantings to reduce pest reproductive success depends on each insect's host range. Clonal hybrid poplar cuttings have helped provide some insights into the relationship between host plant chemistry and insect feeding breadth. The principal secondary compounds present in poplar, phenolic glycosides, have been shown to reduce Lepidoptera performance (Lindroth and Hemming 1990). Ecological theory predicts that feeding by generalists is usually incited by ubiquitous plant chemicals, such as sucrose, and deterred by specific allelochemicals that can inhibit growth and survival (Dethier 1982). Conversely, specialists can metabolize the toxins produced by a particular plant species, and are often attracted to these allelochemicals. For example, feeding by the cottonwood leaf beetle, a specialist on *Populus*, is incited by tremulacin (Bingaman and Hart 1992 a,b). Thus, ecological theory predicts an inverse order of preference among clones by the forest tent caterpillar, a moderate generalist, and the cottonwood leaf beetle. Assays conducted by Robison and Raffa (1992a) confirmed this relationship.

Although most work on plant-insect interactions has concentrated on chemical defenses, recent attention has included plant architecture (Floate and DeClerk-Floate 1992). Evidence from natural systems suggests that certain plant forms can either support or restrain insect performance. Again, it is difficult to rigorously test such theories under controlled conditions. However, hybrid poplar clones provide a wide diversity of plant morphology, which could facilitate testing of these ideas. Among our 15 clones, for example, the ratio of tree height to diameter varies by a factor of over 2X, the ratio of bole to branch dry weight varies by nearly 2X, and the length of secondary stems can vary from 3.2 m to 0 m after 2 years (Robison and Raffa 1992a). The value of this system as an experimental model is heightened by the fact that both insect resistant and insect susceptible clones occur among each category, which facilitates comparative studies.

A final illustration of how vegetatively propagated trees can contribute to our understanding of plant-insect interactions concerns the multiple functionality of allelochemicals. Earlier models of plant-insect coevolution assumed a relatively simplistic interaction, in which single insect species were selected for by patterns of plant chemistry. Recent studies have attempted to develop more inclusive models that consider the relative impacts of multiple selective pressures (Bernays and Graham 1986, Price 1990). Evaluations of hybrid poplars (Robison and Raffa 1992d) revealed clonally based allelopathic weed suppression. Interestingly, one such clone also exhibits high resistance to Lepidoptera, *Septoria*, and rodents. Preliminary evidence suggests that one chemical group, phenolic glycosides are at least partially involved in protection against this broad array of biotic stress agents (Robison and Raffa 1992 a,b,d). This interpretation seems compatible with models of evolutionary parsimony (Bazzaz et al. 1987), whereby organisms produce the fewest necessary amounts of metabolically costly materials. Future research is needed on this question, but the clonal *Populus* model will continue to be extremely useful.

Contributions of Vegetative Propagation to Insect Resistance Breeding Programs

Breeding for insect resistance is confounded by the need for an optimal combination of traits, not just the ability to avoid damage by one particular species (Hanover 1975, 1980). Insect resistance must be kept in perspective. For a particular species or clone to be practical, it must first have good rooting properties, adapt to the particular soil conditions under which it will be grown, be drought- and temperature-tolerant, grow rapidly, possess desirable form and fiber qualities, and show overall high survival (Libby 1973, Wilkinson 1973, Cannell 1978). Likewise, within the general area of pest resistance, there is little value to planting trees that are simply immune to one target species. Under field conditions, trees are exposed to a broad range of microbial, insect, vertebrate, and weed pests. Even "resistance" to a particular pest species needs to be further delineated according to toxicity, growth inhibition, tolerance, and (with animals) behavioral deterrence.

The need for a complex of desirable traits is a formidable obstacle for tree improvement. The time, size, and variability constraints described earlier are magnified when a specified group of properties is required. Vegetative propagation methods such as grafting, rooting, cuttings, and tissue culture can expedite this process, particularly if integrated with an ongoing sexual crossing program. Research operations still require a large amount of time and effort, but an otherwise insurmountable task can be made feasible.

Burdon (1982) listed the major uses of vegetative propagation in breeding programs as preserving genomes, providing clonal seed orchards for mass propagation, directing mass propagation of trees with full use of non-additive genetic variation, providing genetic information, and providing material for physiological research. Insect resistance studies fall mostly within the last application. We will refer to our work with hybrid poplar clones to illustrate how a large number of traits can be screened fairly rapidly.

Hybrid poplar clones in our study showed a high range of performance in the field, with survival ranging from 97% to 0%. Height growth varied by a factor of approximately 2X, but tolerance rankings, measured as relative growth rates following a controlled level of defoliation, are distinct from primary growth rates. In general, there is an inverse relationship, but two clones deviate from this pattern (Robison and Raffa 1990). These two clones offer the combined features of rapid growth, high survival, and defoliation tolerance.

No such compatibility appears available for combining resistance to the forest tent caterpillar and cottonwood leaf beetle. There is generally an inverse relationship between resistance to these two groups, with the useless exception of two clones that are susceptible to both (Fig. 1A). No clones fall in the region, marked by a triangle, that represents resistance to both defoliators. The best combination occurs with a clone ranking fourth most resistant to the cottonwood leaf beetle, and fifth most resistant to the forest tent caterpillar. Thus, there appears little chance of obtaining combined resistance, especially considering the opposing responses to phenolic glycosides described above. Conversely, there does appear to be potential for combined resistance to Lepidoptera and rodents (Fig. 1 B). Although there is no statistically significant trend overall, three clones fall within the top four groups most resistant to both pests. These results can be further expanded by considering other pest combinations. There is generally an inverse relationship between cottonwood leaf beetle and *Septoria* canker resistance (Fig. 1C), and no clones have a combined ranking better than fourth and fifth, respectively. A second opportunity for combined resistance appears to arise with *Septoria* canker and Lepidoptera. Despite the lack of overall relationship, three clones ranked at least in the top five for resistance to both pests (Fig. 1D).

Thus, the most promising clones, along with their shortcomings, can be rapidly identified. This can provide strong guidance for tree improvement programs. For example, we can plan ahead for the likelihood that any clone with resistance to Coleoptera will require additional protection from Lepidoptera, rodents, and *Septoria*. This protection could be provided through tolerance, transgenic resistance, or an Integrated Pest Management program targeted to these pests (Oliveria and Cooper 1977, Caldbeck et al. 1978, McNabb et al. 1982). Conversely, clones with Lepidoptera resistance will probably require special efforts against Coleoptera, but there is good potential that they can be resistant to *Septoria* and rodents. Fortunately, at least one highly productive clone is associated with resistance to each pest (Robison & Raffa 1992 a,b).

A second major area in which vegetative propagation can facilitate insect resistance research is in the application of biotechnology, or more specifically, the integration of biotechnology with traditional breeding methods. Direct transfer of insect resistant traits can provide protection to normally susceptible clones (McCown et al. 1991a, Robison et al. 1992). For example, expression of Bt endotoxin in clone NC5339, protected seedlings against gypsy moths and forest tent caterpillars (Fig. 2). Related Bt genes are effective against cottonwood leaf beetles (Bauer 1990, Ramachandran et al., in press), but these have not yet been inserted into trees. Benefits from genetically engineered resistance could include reduced pesticide applications, and the increased economic competitiveness of biomass alternatives to fossil fuels.

However, a number of ecological dangers could accompany widespread deployment of such toxins (Gould 1988, Raffa 1989). As with pesticides, resistant cultivars, or clones (McNabb et al. 1982),

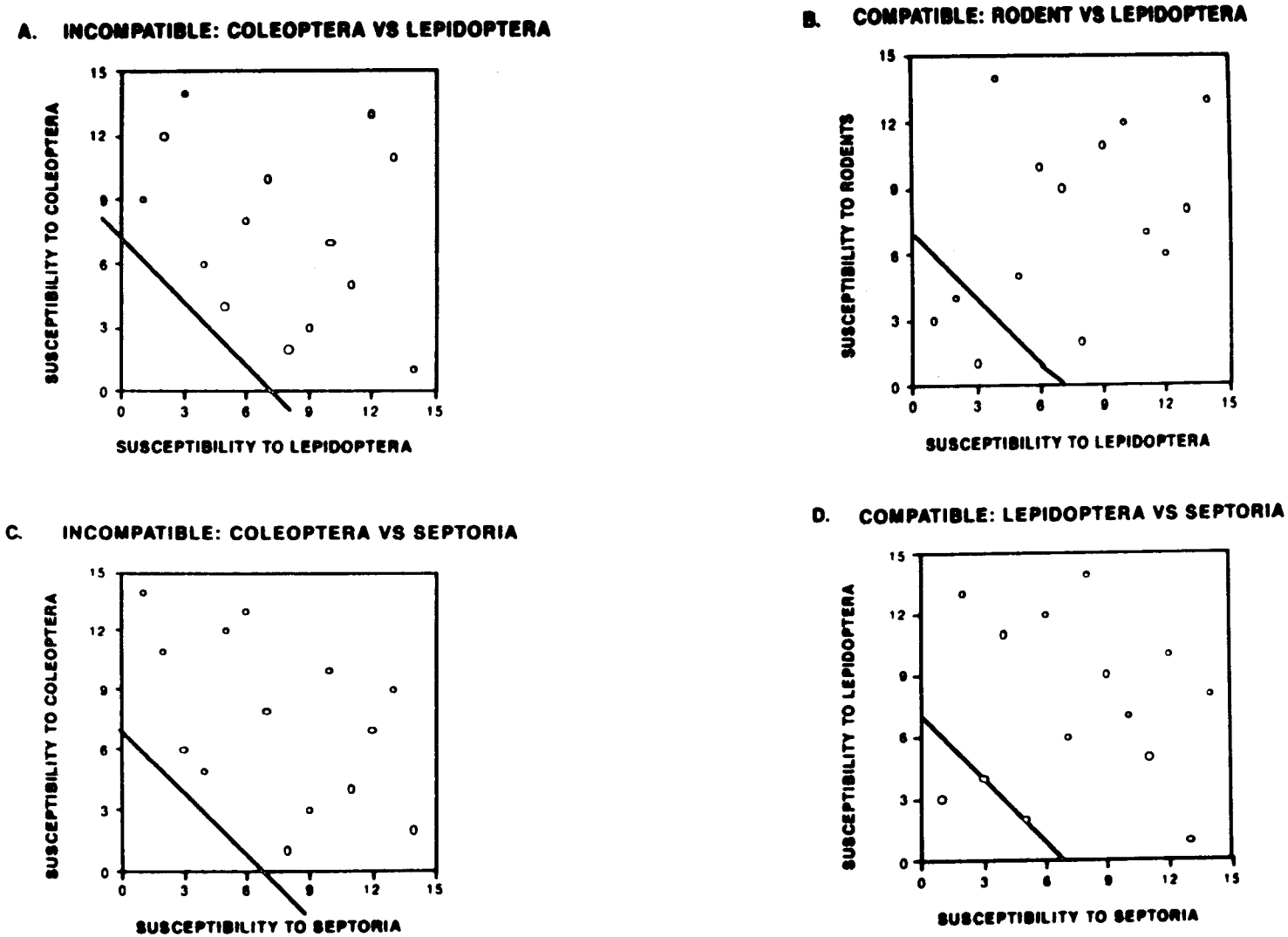

Figure 1. Combined resistances among 15 hybrid poplar clones. For each figure, the rank order of susceptibility (1 = least susceptible) to one pest species is plotted against the same rank order to a second pest. (Data from Robison and Raffa 1990, 1992a,b,c,d). The lines connect the seventh most resistant clone to each pest, and so illustrate zones in which both rankings would have to occur for combined resistance. Some combinations illustrate the possibility for compatible pairs (B,D), while others (A,C) show reveal only incompatible resistance combinations.

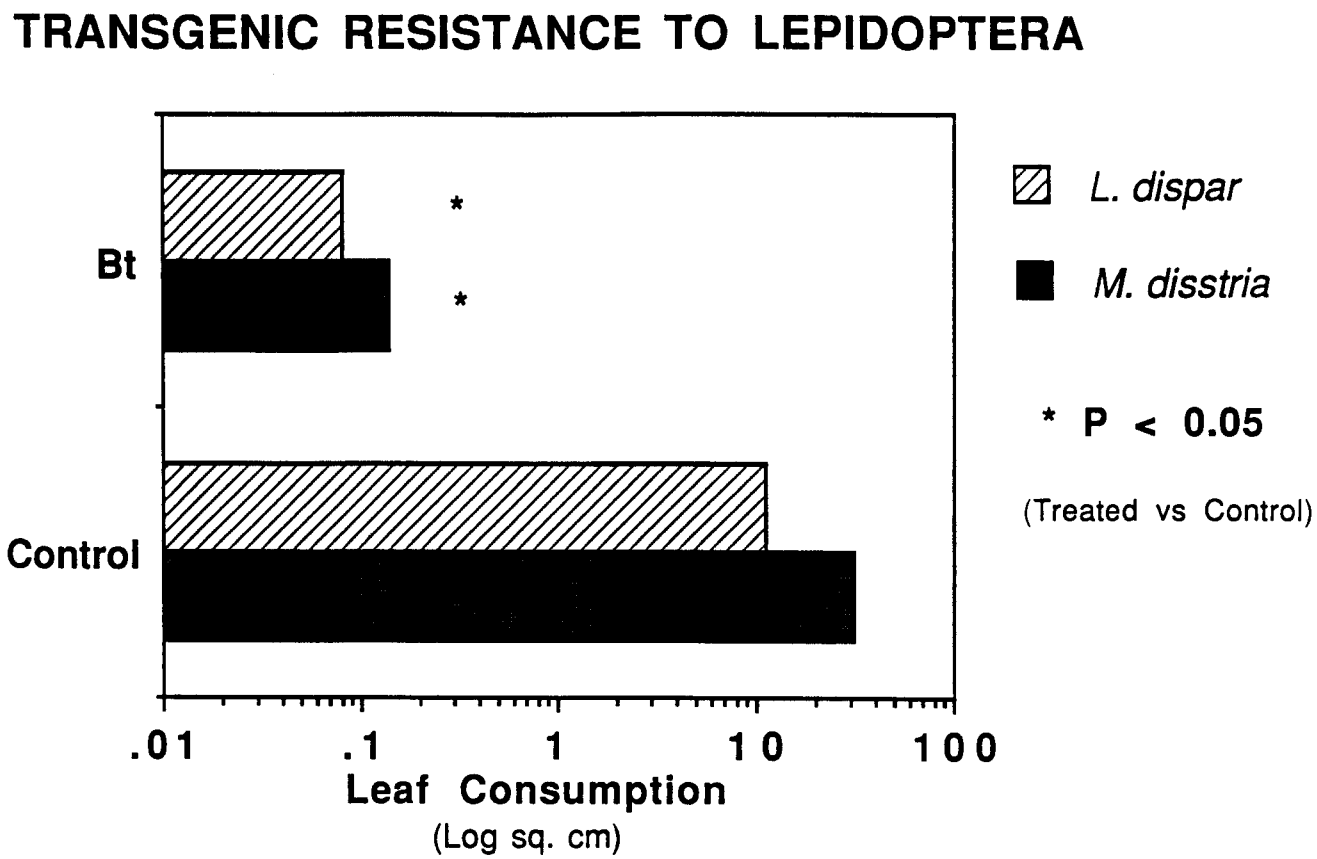

Figure 2. Transgenic resistance against *Lymantria dispar* and *Malacosoma disstria* in poplar seedlings expressing Bt endotoxin (Data from McCown et al. 1991a).

any widespread deployment can favor resistant biotypes, reduce natural enemy populations, and indirectly cause secondary pest outbreaks. Very carefully conceived deployment strategies are needed to manage these potentially adverse effects.

The sheer number of variables that must be considered to accurately assess the environmental safety of genetically engineered trees could delay development of this potentially beneficial technology. Vegetative propagation can greatly expedite this process. For example, our group's current efforts are aimed at developing planting mosaics, that involve mixtures of clonal and transgenic resistance (McCown et al. 1991, Raffa et al. 1992, Ramachandran et al. 1992 a,b; Robison et al. 1992 e). Within this framework, only the Bt gene active against Lepidoptera would be inserted into beetle-resistant clones, and only the Coleoptera-active Bt would be placed into Lepidoptera-resistant clones. Some less resistant, but highly tolerant, clones might be included in these plantings to provide reservoirs for the susceptible insect gene pool. We have also proposed that within-plant spatial, temporal, and wound-inducible expression might reduce biotype evolution and secondary pest eruptions (Raffa 1989).

Vegetatively propagated plants are helping to transfer this theoretical framework into reality. For example, we can simulate the patterns of within-plant expression described above, by artificially applying appropriate Bt extracts to foliage. Once we evaluate the relative value of each tactic, molecular biologists can prioritize their efforts accordingly. For example, application of Bt to only the young foliage causes beetles to feed only on older foliage (Ramachandran et al., in prep). This does not provide complete plant protection, but can reduce damage substantially, and allows some Bt-susceptible beetles to survive.

Vegetatively propagated research material can also increase the efficacy of transgenic plants. Interactions between Bt and host allelochemicals can greatly affect toxicity, for example (Krischik et

al. 1988). Thus, a judicious choice of clones can synergize interactions between leaf chemistry and transformed properties.

CONCLUSION

Vegetative propagation can greatly expedite research on insect resistance in trees. In some cases, the relative ease of propagation could determine whether or not a particular objective is achievable, given practical constraints on time and resources. Research fostered by vegetative propagation has far reaching implications to basic ecology and evolution, the development of high-yielding insect-resistant trees, and the environmentally safe implementation of genetic engineering.

TREE PROPAGULE/MICROBE GENETIC INTERACTIONS

In our forest pathology project we use vegetative propagules for a variety of purposes. Our mission is to develop systems for rapidly imparting resistance to specific diseases of forest trees. This program, which started about seven years ago uses the phenomenon of somaclonal variation to obtain genetic change without going through the long process of traditional sexual breeding.

Somaclonal variation is the genetic variation exhibited by plants grown in aseptic culture. Although the causes of somaclonal variation are not completely understood, the phenotypic and genetic variation found in various plant crops indicates that many factors are involved. These include minor point mutations, polyploidy, transposable elements, aneuploidy, and epigenetic changes. Somaclonal variation can result from preexisting genetic variation that is expressed in regenerated plants, or it can be induced during the tissue culture process itself (Scowcroft and Larkin 1983).

I would like to give a few examples of how vegetative propagules have helped our research program at the North Central Forest Experiment Station.

Hybrid poplar-Septoria canker.--Our original research with the process of somaclonal variation was with hybrid poplar. The study was conducted to demonstrate the potential application of somaclonal variation for increasing disease resistance in a tree species. *Populus* was chosen because of its widespread importance as a source of fiber and energy. This genus is also conducive to whole plant regeneration by a number of vegetative propagation systems including tissue culture. Yields from hybrid poplar plantations are seriously limited by the foliar and canker diseases caused by the fungus *Septoria musiva* Peck. Our objective was to take high-yielding hybrid poplar clones and increase their resistance to *Septoria* canker. Callus cultures of susceptible clones were initiated from stem internode explants taken from greenhouse plants. These callus cultures were maintained for 5 to 13 months before shoot proliferation was induced.

Elongated shoots were excised and rooted before they were placed in the greenhouse. New, fully expanded leaves were collected from these plants and inoculated with conidia of *S. musiva* on the abaxial surface. Plants with increased resistance to Septoria were recovered from two of the three previously susceptible clones. Many regenerates were significantly more resistant to *Septoria* canker than were the parent plants (Ostry and Skilling 1988). These putative resistant plants have now been field-tested for resistance for 6 years. Many of these plants continue to show resistance to *Septoria* canker. The best selections have been asexually propagated for more extensive field evaluation. Although the field testing of this plant material requires several years, we were able to produce a number of *Septoria*-resistant plant lines within a year after starting this study. In this situation, using vegetative propagules has been very productive both in the final results and in the shortened time needed to produce these results.

European larch - Gremmeniella canker.--The first attempt to produce somatic variants in a conifer species was with European larch (*Larix decidua* Mill.). Our objective was to produce resistance to *Gremmeniella* canker caused by the fungus pathogen *Gremmeniella abietina* (Lagerb.) Morelet. In this model system the cotyledon culture technique was used to produce somatic variants with resistance to *G. abietina* (Diner et al. 1986). The resulting plantlets were inoculated in vitro with conidia of *G. abietina*. Plantlets that survived three inoculations were then multiplied both in vitro and ex vitro from axillary buds to produce clonal lines with putative resistance. At present we have 50 clonal lines that are being field-tested in Wisconsin and New York for resistance to *G. abietina*.

American chestnut - Chestnut blight.--One of our cooperators, Dr. Dennis Fulbright, at Michigan State University, has identified American chestnut (*Castanea dentata* (Marsh.) Borkh.) germplasm with high levels of resistance to chestnut blight caused by the fungus *Cryphonectria parasitica* (Murr.) Barr. Dr. Fulbright is developing in vitro propagation techniques for multiplication of these disease resistant trees. This research has progressed to the point where the plantlets from resistant trees are rooting very well, although survival is reduced when the plants are placed on a peat-perlite mixture. He is also developing in vitro inoculation techniques for screening germplasm prior to field planting. This technique saves both time and money. In this study the use of vegetative propagules will insure that our germplasm orchards will maintain plants with the original genes for resistance. Whether this propagation system can produce asexual plant material at a reasonable price per unit is not known.

Butternut - butternut canker.--The last example I want to present is our research on the development of butternut (*Juglans cinerea* L.) with resistance to butternut canker caused by the fungus *Sirococcus clavigignenti-juglandacearum* Nair, Kostichka, and Kuntz.

Forest populations of butternut trees are rapidly being lost as a result of this fungus. Because of this disease, butternut is being considered for the endangered species list. Our program uses a three part approach to produce resistant butternuts. The first part is the establishment of grafted clonal lines of butternut from putative resistant trees. These are inoculated with the butternut canker fungus to determine resistance. We now have 20 clonal lines that are being evaluated. The second part of the program is to develop techniques to rapidly propagate resistant lines from these grafted trees. The objective here is to maintain the resistant genes in the asexually produced progeny. Rooting of butternut plantlets from axillary buds has been accomplished with juvenile material. Our next step will be rooting of shoots from the grafted plants. The third part of this project is to look for somatic variants that may have canker resistance. In this work we are collecting immature butternut during the month of July. Our results indicate that immature cotyledonary tissue of *J. cinerea* is amenable to successful induction of somatic embryogensis. Work is continuing on development of entire plants from this embryogenic material. If this is successful, it would provide us with an additional source of butternuts that may have resistance to this serious canker disease.

From the examples that we have included in this paper, it is obvious that vegetative propagules have an important role in the research arena. In many cases they supply us with additional opportunities to develop new plant material or to conduct more sensitive tests on existing plant material. They have limitations and must be used with caution, but the system offers both speed and the ability to produce large numbers of resistant clones. Tree improvement is a difficult process under the best of conditions. Anything that will increase the success of this work has real value. We feel that vegetative propagules can contribute to this success.

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