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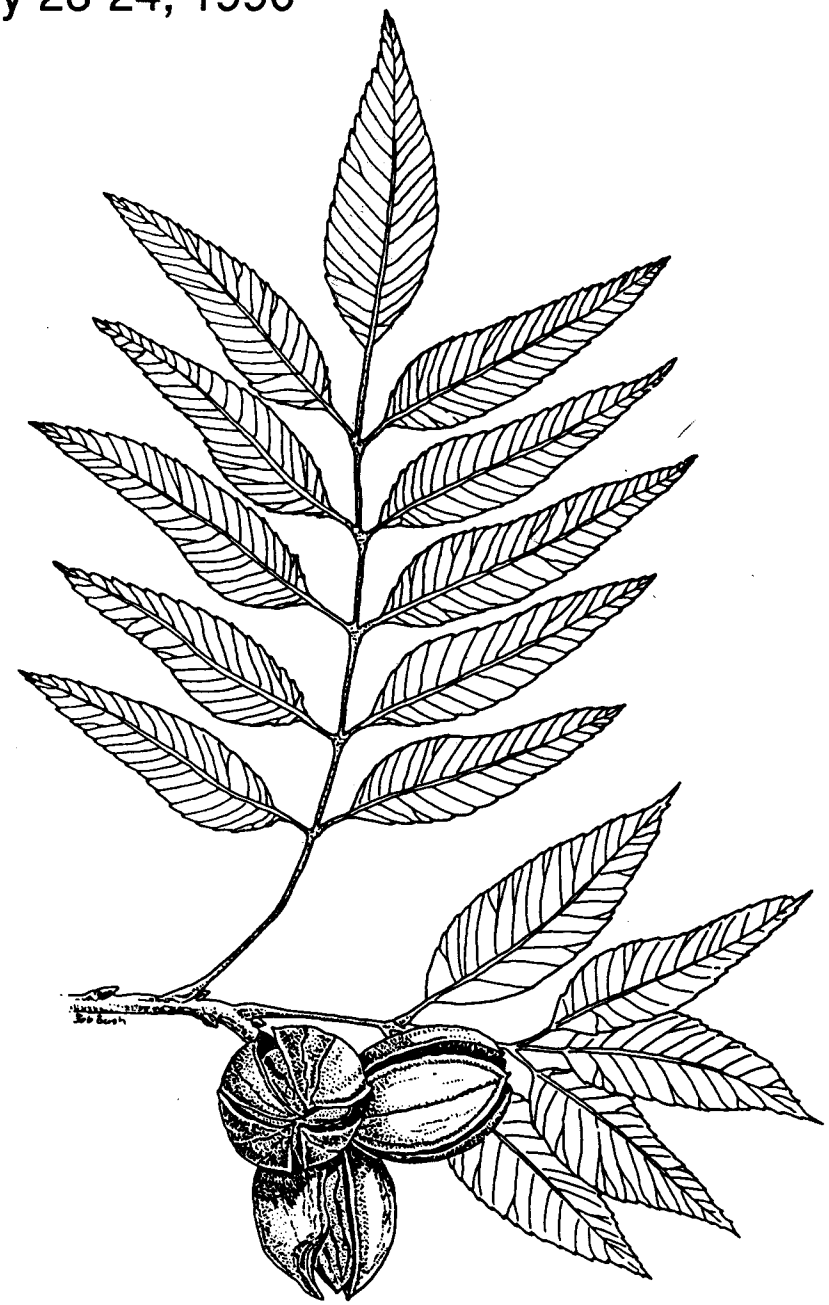

TISSUE CULTURE OF PECAN

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Plant tissue culture had its origins in the 1930's as an academic endeavor to understand developmental mechanisms in plants. As true with other areas of plant science, the objectives of plant tissue culture have evolved, in many cases, to product-oriented goals. Examples of plant tissue culture techniques being explored for commercialization are micropropagation; generation of genetic variants either through tissue culture alone or in conjunction with gene transfer technologies; encapsulation of somatic embryos for seed dispersal; germplasm preservation; and production of plant secondary metabolites for use in the pharmaceutical, food-processing, and pesticide industries (Bock and Marsh 1988). Most research in pecan tissue culture has focused on either micropropagation or development of genetic variants and this paper is a review of these two subjects. The products generated through tissue culture in the 1990's can be expected to increase as progress is made in the refinement of the basic technologies.

MICROPROPAGATION

Micropropagation is a term usually restricted to the mass propagation of clonal plants in culture; i.e., the production from a single plant part of numerous plants each being genetically identical to the mother tissue. One of the most common methods of micropropagation is to force a bud, either excised from or still attached (nodal segment) to the stem, to produce many shoots instead of only one. The shoots are then treated with hormones to induce rooting.

Micropropagation was the first area of plant tissue culture to be of commercial value. The orchid industry was one of the commercial enterprises to benefit first from the commercial applications of micropropagation. Prior to the 1960's, conventional vegetative propagation techniques for orchid production were very slow. In a series of orchid tissue culture studies designed to eliminate viruses, the relatively short time required for the generation of clonal orchid plants was realized quickly (Rao 1977). This caused a revolution in the orchid industry. By 1984, there were over forty companies in the U.S. and many others throughout the world specializing in orchid production *via* micropropagation (George and Sherrington 1988). This and other success stories, primarily with ornamental plants and a few vegetable crops, led to the concept that thousands of clonal plants of any given plant species could be obtained within a few months by excising the appropriate plant part and providing the excised plant part with the appropriate balance of phytohormones.

Skepticism is mounting that plants generated *via* micropropagation can be considered to possess absolute clonal fidelity because somaclonal variation is known to occur (Scowcroft and Larkin 1988). The term somaclonal was coined originally to describe all plants arising from the same initial piece of explant material. It was assumed that all plants would be genetically identical; however, this may not be true, even for plants in which there is no intervening callus phase. Hence, the term somaclonal variation evolved. However, the following discussion is written on the premise that micropropagation will preserve the genetic identity and integrity of the mother tree as faithfully as conventional propagation techniques.

Advantages in Micropropagation of Pecan

Micropropagation of pecan would be advantageous for rapid clonal multiplication and germplasm preservation. Rapid clonal multiplication would be useful in studying elite pecan trees with desirable characteristics and rootstock influences on tree productivity. For example, hundreds of plants could be produced within a few months from a seedling tree selected for resistance to insects or diseases. These plants could be tested at different geographic locations to determine whether the characteristics of interest remained constant under various climatic, insect and disease pressures. Likewise, micropropagation could provide genetically identical plants for investigating the control by the rootstock on tree productivity.

Another advantage in being able to micropropagate pecan would be for germplasm preservation. Techniques for the integration of micropropagation

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with germplasm preservation usually involve cryopreservation of buds that are then regenerated to plants as needed or, alternatively, maintenance of culture stocks on medium that dramatically reduces the growth rate so that infrequent transfers are required. With cryopreservation techniques using liquid nitrogen, up to four buds from each of 684 trees could be stored in a tank 5 feet in diameter and 2 feet in height. Considering there are over 1,000 named pecan cultivars (Thompson and Young 1985) and untold numbers of seedling sources, maintenance of the tremendous genetic variability that exists in the population of pecan in the U.S. by conventional field propagation techniques is a forbidding task. Even through tissue culture, such a task would be unreasonable. However, the percentage of the available types that could be maintained would be increased. The dollars saved over tree maintenance in the field would be impressive.

Advances in Pecan Micropropagation

The first reported attempt at tissue culture of pecan was not until the late 1970's (Smith 1977, Smith and Storey 1977) fairly recent when considering the science of woody plant tissue culture began more than 50 years ago. Smith and Storey (1977) cultured seedling pecan stem segments on a variety of media formulations. Callus was initiated and one or two spontaneous roots and/or shoots were produced using IAA and kinetin in relatively low concentrations (0.75 to 1.1 ppm IAA with 1.1 ppm kinetin for roots, 0.75 ppm IAA with 2.0 ppm kinetin for shoots). Best growth of callus was between 0.5 to 1.0 ppm IAA and kinetin. Knox and Smith (1978) produced a shoot and root in tissue culture, but technical details were not defined. Knox (1980) described obtaining plants by culturing nodal segments from 'Riverside' seedlings first on medium containing 0.1 to 0.7 ppm IBA and 0.1 to 2 ppm BA followed by culturing on 1 ppm IBA and 1 ppm phloroglucinol. Wood (1982) reported the first successful multiple shoot proliferation from nodal segments. Hansen and Lazarte (1984) described obtaining roots on shoots multiplied in culture. In order to circumvent the problems of contamination as described by Wood (1982), zygotic embryos were dissected from the developing fruit and germinated *in vitro* for multiple shoot proliferation (Yates and Wood 1989, Ou 1989). Rooting of multiple shoots generated in this manner was accomplished (Ou 1989).

All the work discussed to this point centered around tissues obtained from seedlings or seeds. Any plants regenerated from these tissues would have a new combination of genes. To be of more immediate commercial application, propagation would be more beneficial from mature pecan trees in which characters of interest have been demonstrated. Plants regenerated through multiple proliferation from buds or meristems would have the same characteristics as the mother plant. Thereby, the 15 to 20 years required to evaluate field performance for pecan plants that contain unknown characteristics could be eliminated. Pecan trees that are 10 to 15 years old may appear to have desirable crop production characteristics, but as the trees reach maturation after 15 to 20 years growth, fruit quality may become unacceptable (Sparks 1990). Thus, plants developing from cross-pollinated seeds or any tissues thereof must be subjected to decades of field tests.

Cotten (1983) attempted to obtain *in vitro* multiplication of shoots from buds of mature pecan trees; however, this study was hampered by contamination and the exudation of phenolics from the explant during culture establishment. Phenolics were reduced by adding charcoal to the medium and incubating the cultures in darkness. Treatment of explants with 70% ethanol for 8 min, then with 0.525% sodium hypochlorite for 15 min followed by a 4.5 hr soak in a saturated benomyl solution was more effective than using antibiotics to control contamination. Even though parameters were optimized for culture establishment, no plants were obtained in these studies.

Recently, plant regeneration has been achieved by axillary bud proliferation of nodal stem segments taken from mature trees (Dr. Gregory Phillips, Department of Agronomy and Horticulture, New Mexico State Univ., Las Cruces, personal communication; Corte-Olivares 1987). However, contamination was a major problem during culture establishment in this study and only an average of one shoot/explant was produced. In our laboratory, we have utilized techniques similar to that used for walnut which involves washing the shoots for 4.5 hr in running water prior to inoculation into culture medium (McGranahan et. al. 1988a). However, with field material collected in the southeastern U.S., such techniques are not stringent enough to eliminate gross contamination of cultures.

In summary, micropropagation of pecan has been achieved with tissues from both juvenile and mature trees. However, more work is required before large numbers of trees can be generated rapidly and repeatedly through these procedures.

Novel propagation techniques will be required to successfully micropropagate pecan from mature trees.

Difficulties in Micropropagation

The principle of micropropagation is simple. However, putting these principles into practice for pecan is not so easy, especially using tissues from mature wood. Micropropagation of seedling material less than one-year-old has been successful in the laboratory, but no plants have been hardened off to grow in the field or even in the greenhouse. There are two major problems encountered in the micropropagation of pecan from field-grown material. One is contamination of the plant cultures with microorganisms, and another is the recalcitrant nature of pecan *in vitro*.

Even though developing repeatable micropropagation techniques for pecan has been slow, so has the progress with trees in general. The protocol suitable for herbaceous plants is not directly applicable to woody plants, especially species of forest and nut trees. Although over five decades have elapsed since the pioneering plant tissue culture studies with tree species in the 1930's (Gautheret 1934), there are still no commercially micropropagated forest tree species in the U.S. (Hanover 1987). This serves to highlight the technical difficulties of establishing, maintaining, and manipulating cultures of tree species. As a result of the long-term commitment required to surmount these difficulties, the interest and financial support for developing micropropagation methods for woody plants is waning (Mezitt 1988).

There are several problems that have limited progress in micropropagation of pecan, as well as many other tree species. These problems include: 1) obtaining appropriate explant material; 2) contamination of explant material; 3) release of toxins from the explant in the medium; 4) low shoot multiplication rates; and 5) poor or no root formation. The first three problems may be encountered during culture establishment.

Due to the nature of the annual cycle of growth, obtaining the appropriate plant part to initiate a culture may be possible for only a few weeks or even a few days out of the year. For example, anthers in pecan are produced on an annual basis and are at the appropriate stage for tissue culture for only a few days. Therefore, the supply of experimental material seriously limits the scope of possible investigations. Another common difficulty is establishing aseptic cultures; i.e., cultures with only one organism growing. With the objective to micropropagate an elite pecan tree selected for its field performance, axillary bud proliferation would be a theoretically suitable methodology. However, such tissues may be difficult to establish in culture because field-grown plants frequently harbor fungi and bacteria which are difficult to eliminate. Stringent surface sterilization procedures used on the explant to reduce the growth of these contaminants in culture may reduce the viability of the plant tissue (Knox 1980). To avoid this problem, antibiotics which are specific for the contaminants may be added to the medium. Such an approach has not been successful in pecan (Cotten 1983). Another problem often encountered in culture establishment is that on excising the explant from the source plant, the injury response generates production of toxins which are detrimental to the growth of the plant tissue (Graves et al. 1988). This response may be partially alleviated by treating the explant with chemicals to retard oxidation processes, using a large explant initially and removing deteriorating portions every few days during culture establishment, or transferring the explant material to fresh medium as discoloration increases. Extensive browning of tissues, as well as diffusion of these chemicals into the culture medium, may retard or prohibit the growth of the explant tissue.

Once culture establishment has been achieved, the next challenge is the activation of growth of the appropriate plant part at the appropriate site on the explant. The appropriate stimuli for shoot elongation and multiplication must be provided *via* culture medium and environmental conditions. Once shoot multiplication has been attained, producing roots on those shoots is another hurdle.

Finally, acclimatizing plants generated *in vitro* from the nutritive conditions in the culture medium to soil conditions is difficult. Survival rates have been low when converting pecan plants regenerated *via* tissue culture techniques from the conditions in the culture vessel to soil conditions. Consequently, accumulating enough plants at any one time to conduct meaningful experiments to determine the appropriate protocol for hardening off plants has not been accomplished. We do have a few plants in the greenhouse that have been carried through tissue culture, but not in a large enough quantity from any one experiment to be able to give definitive techniques for establishing protocol.

GENERATION OF GENETIC VARIANTS

Genetic variability *per se* would not appear to be a primary objective for pecan tissue culture. With over 1,000 named cultivars and millions of seedlings existing in the wild, we do not have the facilities for preservation of the genetic variation already existing. Seedlings surviving in nature probably have much adaptive conditioning already inherent in their genome (Sparks 1990). Association of this variability in the proper combinations by directed gene transfer is the primary value of this area of plant tissue culture for pecan. A seedling pecan tree or cultivar may exist that has appropriate adaptive growth characteristics for the environmental niche in which it has survived thus far (Sparks 1990). However, one or two characteristics may be lacking that could improve crop production. For example, 'Desirable' produces a nut that brings a good price, alternate bearing is minimal, but early fruit maturity would enhance marketability. Identifying the part of the genome responsible for early fruit maturity in another cultivar or seedling selection and transferring this to 'Desirable' would produce a highly profitable cultivar for the southeastern U.S. (Sparks 1990).

Improvement of Pecan through Genetic Variation

Techniques of pecan tissue culture have been developed that will provide for the application of directed gene transfer for pecan tree improvement. One factor responsible for this is the ease with which somatic embryos can be generated from pecan tissue culture (Laird 1985, Yates and Corte-Olivares et al. 1990, Yates and Reilly 1990). Somatic embryos are different from zygotic embryos. Zygotic embryos are produced by the classical reproductive scheme of the union of the egg and sperm. "Soma" is the Greek word for body; hence, somatic embryos originate from body cells, not reproductive cells.

Developing techniques for somatic embryogenesis in pecan has proven to be a much easier system of plant culture to manipulate than micropropagation from stem segments. One reason for this is that establishing cultures free of microorganisms is not a problem. In most cases, the initiating tissue is derived from the developing nut. In our laboratory, the protocol is to soak the fruit (husk and ovary) in 70% ethanol for 20 min. Subsequently, the husk is discarded and the embryonic tissue is placed in culture. This tissue is already in a metabolically active mode to form cotyledons so that activation of growth is not a problem. Thus, both the problem of contamination and of growth activation encountered in micropropagation techniques are circumvented. Somatic embryos are expected to be useful in directed gene transfer. McGranahan et al. (1988b) have been able to utilize walnut somatic embryos to demonstrate direct gene transfer. Such techniques should be directly applicable to pecan since the vehicle used to infect walnut embryos is *Agrobacterium*, the organism responsible for crown gall in pecan roots. Directed gene transfer experiments are currently in progress with pecan.

Difficulties of Introducing Genetic Variation into Pecan

In the early 1970's and 1980's, immediate commercial gains by the methods of biotechnology were considered to be achievable for plant improvement within a few short years. A quote by Wilson and Sullivan (1984) in Economic Review reflects the optimism inherent in those times: "Civilization may stand on the edge of a biotechnological revolution that could affect virtually every area of the environment". These authors predicted that a number of plants with minor improvements could reach the marketplace by 1987 and plants with significant genetic alterations would be commercially available by 1990. These predications have not come to fruition.

Independent commercial biotechnology companies were formed with the view of becoming profit-making enterprises. Companies with names tying their production objective to genetic engineering (Genetex, Calgene, and Agrigenetics) were formed. Agrichemical companies such as Monsanto and Dupont invested large amounts of capital to establish in-house research programs in biotechnology. Established companies not setting up their own in-house research programs financed research through biotechnology companies. For example, Kellogg, a major food processing company, invested $10 million in Agrigenetics Corporation in 1982 (Wilson and Sullivan 1984). For this investment, Agrigenetics was to develop strains of oats, rice, and wheat with high protein content and minimize fertilizer requirements for the growth of these plants. The financial returns expected by Kellogg did not materialize. Important results on the molecular biology of these organisms did emerge, but the outcome was still at the purely basic biology level and not of commercial application.

The priorities set by the Agriculture Research Service in the mid-1980's are other examples of the optimism that biotechnology was going to be a quick fix for developing superior plants. A brochure entitled "Solving Agricultural Problems with Biotechnology" was issued. In this brochure, yearly ARS funds of $26.7 million were identified as being directed to biotechnology, and a long-term commitment to biotechnology was emphasized. By the late 1980's, skepticism of the immediate commercial exploitation of this science became apparent as funds were redirected to other research priorities.

The application of genetic engineering to pecan, as well as other plant species, has been hindered by two major problems. One is that isolation of the desired genes has been limited because the biochemical or genetic basis of the phenotype is unknown. Another is that plant cells are much less amenable than animal cells to molecular biology studies. The restricted adaptability of plant cells for molecular studies is due to two parameters. One is that plant cells have a cell wall which restricts the introduction of foreign materials into the cell. The other is that genetic analysis is complicated because some plant characteristics are controlled outside the nucleus by chloroplast and mitochondrial genomes.

In summary, the immediate need for genetically engineering pecan trees is to conduct fundamental studies on biochemistry and molecular biology. These studies are necessary to identify genes determining the phenotypes of characteristics considered desirable in a commercially productive pecan tree. Important genes then may become targets for genetic engineering. In addition, information on the regulation of gene expression will be required in order to know how to turn these genes on and off in the right place at the right time. The belief is still alive that the timing and extent of plant developmental processes such as flowering, seed formation, ripening and senescence may one day be changed through genetic engineering (Grierson and Covey 1988).

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