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INVITED REVIEW

Biotechnological efforts for preserving and enhancing temperate hardwood tree biodiversity, health, and productivity

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Abstract Hardwood tree species in forest, plantation, and urban environments (temperate regions of the world) are important biological resources that play a significant role in the economy and the ecology of terrestrial ecosystems, and they have aesthetic and spiritual value. Because of these many values of hardwood tree species, preserving forest tree biodiversity through the use of biotechnological approaches should be an integral component in any forestry program in addition to large-scale ecologically sustainable forest management and preservation of the urban forest environment. Biotechnological tools are available for conserving tree species as well as genetic characterization that will be needed for deployment of germplasm through restoration activities. This review concentrates on the biotechnological tools available for conserving, characterizing, evaluating, and enhancing hardwood forest tree biodiversity. We focus mainly on species grown for lumber and wood products, not species grown mainly for fiber (pulp and paper production). We also present a brief summary of the importance of non-wood forest products from temperate hardwood tree species (a research area that needs further development using biotechnological techniques) and a few case studies for preserving forest tree biodiversity.

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Introduction

Hardwood tree species in forest, plantation, and urban environments (and other temperate regions of the world) are important biological resources that play a significant role in the economy and the ecology of terrestrial ecosystems, and they have aesthetic and spiritual value. The hardwood tree resource provides timber for lumber and veneer production used in the manufacture of residential and commercial structures, interior furnishings, and numerous specialty wood products (e.g., baseball bats, basketry, billiard cues, golf club heads, musical instruments, tool handles, etc.; Forest Products Laboratory 1999; USDA-NRCS 2010); fuel wood; and raw materials used in the manufacturing, drug, cosmetic, and botanical industries (Ciesla 2002; Jones et al. 2002; USDA-NRCS 2010). Hardwood trees provide environmental stabilization as riparian buffers, windbreaks, watershed protection and in reclamation, restoration and conservation areas (Allen et al. 2001; Jacobs 2006; Skousen et al. 2009). Trees in the urban forest help conserve energy and control pollution, provide habitat for urban wildlife, and provide pleasing recreational and ornamental settings in parks, nature preserves, and other urban gathering places. Many hardwood tree species provide shade, shelter, nesting sites, and nutritional food for wildlife (Leopold et al. 1998; DeGraaf 2002; MacGowan 2003; USDA-NRCS 2010). The mixed hardwood species composition in natural forests (Burns and Honkala 1990) provides additional complex flora, fauna, and soil associations. Dying and dead

hardwood trees, whether standing, fallen, or at different stages of decay provide valuable habitats for insects, invertebrates, lichens, birds, and mammals, as well as play an important role in nutrient cycling and moisture retention (Harmon et al. 1986). Hardwood trees also provide nutritional value (nuts and sap for syrup) for human consumption, are aesthetically pleasing in the forest and urban landscape, and historically they have provided a spiritual component in human culture (Ciesla 2002; Jones et al. 2002). Biodiversity, in the context of forestry, can then be defined as including the genes, individuals, populations, species, communities, and the interaction between these components (Lindenmayer and Franklin 2002). Because of the high value of hardwood tree species, preserving forest tree biodiversity through the use of biotechnological approaches should be an integral component in any forestry program, in addition to large-scale ecologically sustainable forest management (Lindenmayer and Franklin 2002) and preservation of the urban forest environment (Alvey 2006). Biotechnology is needed because genes (traits) for various pest and pathogen tolerances do not exist in breeding populations. Some of the more valuable hardwoods include alder, ash, basswood, beech, birch, black cherry, black locust, black willow, chestnut, elm, gum, hackberry, hard (and soft) maples, hickory, pecan, oak, sassafras, sycamore, tuliptree, and walnut. Hardwood tree species such as *Castanea dentata* (Marsh.) Borkh. (American chestnut), *Ulmus americana* L. (American elm), and several *Fraxinus* spp. [*Fraxinus pennsylvanica* Marsh., *Fraxinus americana* L., and *Fraxinus nigra* Marsh.] (green, white and black ash) are threatened because of exotic insects and diseases. Biotechnological tools are available for conservation of these species, as well as genetic characterization that will be needed for deployment of these threatened populations through restoration activities. The role biotechnology can play in plantation forests has been reviewed (Fenning and Gershenzon 2002). Other reviews have focused on innovative technologies that provide the basis for acceleration in forest tree improvement (Nehra et al. 2005) and *in vitro* culture, cryopreservation, gene transfer, and genomics for a number of hardwood timber and pulp species (Merkle and Nairn 2005; Merkle et al. 2007; Pijut et al. 2007; Durkovic and Misalova 2008). This review continues with the literature of completed reviews and concentrates on the biotechnological tools available for conserving, characterizing, evaluating, and enhancing hardwood forest tree biodiversity. We focus mainly on species grown for lumber and wood products, not species mostly grown for fiber (pulp and paper production) such as *Populus* and *Eucalyptus*. We also present a brief summary of the importance of non-wood forest products from temperate hardwood tree species (a research area that needs further development using biotechnological techniques) and a few case studies for preserving forest tree biodiversity.

Conservation Through *In vitro* Technologies

Micropropagation. Schaeffer (1990) defined micropropagation as the *in vitro* clonal propagation of plants from shoot tips or nodal explants, usually with an accelerated proliferation of shoots during subcultures. The ultimate goal in a forest biotechnology conservation program is the clonal propagation of mature, elite genotypes, or cultivars; threatened or endangered tree species; or genotypes with known disease or pest resistance. Usually, protocols are first developed using juvenile explants (from seeds, seedlings, or young trees) to establish methods useful in the development of clonal propagation systems for mature trees. Several species of valuable temperate hardwoods have been successfully propagated using explants from grafted (mature scions) plants or mature trees. Micropropagation of some of these hardwood tree species has been reviewed (Merkle and Nairn 2005; Pijut et al. 2007; Durkovic and Misalova 2008), and recent developments will be discussed here (see also Table 1).

A Czech cultivar of Norway maple (*Acer platanoides* L. "Jirka"), suitable for planting in urban areas because of its compact form, was micropropagated from 5-y-old trees (Sediva 2009). Nodal explants cultured on woody plant medium (WPM) (Lloyd and McCown 1981) with 20 g L^{-1} sucrose and 2.28 μM zeatin produced shoots suitable for rooting (63%) on half-strength WPM containing 49.2 μM indole-3-butyric acid (IBA). Micropropagation of European white birch (*Betula pendula* Roth.), an economically important tree species in Nordic countries and frequently planted in the North American landscape, has been successful (Haggman et al. 2007; Ryynanen and Aronen 2007). Dormant shoot tips or vegetative buds cultured on Murashige and Skoog (MS) medium (Murashige and Skoog 1962), N6 medium (Welander 1988), or WPM containing various concentrations of 6-benzyladenine (BA) and α-naphthaleneacetic acid (NAA) have been used successfully to establish shoot cultures, with subsequent rooting of shoots on MS or WPM with or without IBA. *Betula papyrifera* Marsh. "Varen" (paper birch) and *Betula platyphylla* Sukaczev "VerDale" (Asian white birch), with demonstrated resistance to the bronze birch borer for more than 25 y, have been micropropagated using WPM with 10–20 μM BA or 4–8 μM thidiazuron (TDZ; Magnusson et al. 2009). European chestnut (*Castanea sativa* Mill.), an important timber and nut-producing hardwood, has been propagated using shoot tips and nodes taken from seedlings or basal shoots and stump sprouts of mature trees (Vieitez et al. 2007). This species was propagated *in vitro* on a

Table 1. Recent advances in cryopreservation, *in vitro* culture and genetic modification of hardwood tree species

Genus	Research studies	References
Acer	Micropropagation	Sediva 2009
Betula	Adventitious shoot regeneration, cryopreservation, micropropagation, transgenics	Haggman et al. 2007; Meier-Dinkel 2007; Ryynanen and Aronen 2007; Seppänen et al. 2007; Haggman et al. 2008; Towill and Ellis 2008; Vihervuori et al. 2008; Magnusson et al. 2009; Pasonen et al. 2009; Zeng et al. 2010
Castanea	Cryopreservation, embryogenesis, micropropagation, transgenics	Corredoira et al. 2007; Rothrock et al. 2007; Vieitez et al. 2007; Welch et al. 2007; Corredoira et al. 2008; Goncalves et al. 2008; Lambardi et al. 2008; Roach et al. 2008; Andrade et al. 2009
Fagus	Embryogenesis	Pond 2008
Fraxinus	Adventitious shoot regeneration, cryopreservation, micropropagation, transgenics	Du and Pijut 2008; Chmielarz 2009; Du and Pijut 2009; Mitras et al. 2009; Volk et al. 2009
Juglans	Embryogenesis, micropropagation, rooting, transgenics	Amiri 2006; Caboni and Damiano 2006; Fatima et al. 2006; Leslie et al. 2006; McGranahan and Leslie 2006; Vahdati et al. 2006; Britton et al. 2007; Pei et al. 2007; Rios Leal et al. 2007; Roschke and Pijut 2007; Bosela and Michler 2008; Vahdati et al. 2008; Bahrami Sirmandi and Vahdati 2009; Baya et al. 2009; Hackett et al. 2009; Sharifan et al. 2009; Vahdati et al. 2009a, b; Wang et al. 2009
Liquidambar	Adventitious shoot regeneration, transgenics	Renying et al. 2007; Xu et al. 2007
Platanus	Adventitious shoot regeneration, transgenics	Li et al. 2007; Sun et al. 2009
Prunus	Adventitious shoot regeneration, transgenics	Liu and Pijut 2008; Liu and Pijut 2010
Quercus	Cryopreservation, embryogenesis, genetic fidelity, micropropagation, rooting, transgenics	Neves et al. 2006; Álvarez and Ordás 2007; Celestino et al. 2007; Ostrolucka et al. 2007; Pintos et al. 2007; Santos et al. 2007; Fernandes et al. 2008; Lambardi et al. 2008; Martinez et al. 2008; Pintos et al. 2008; Sanchez et al. 2008; Tamta et al. 2008; Álvarez et al. 2009; Celestino et al. 2009; Gomez et al. 2009; Kartsonas and Papafotiou 2009; Pintos et al. 2009; Vengadesan and Pijut 2009a,b; Vieitez et al. 2009
Robinia	Adventitious shoot regeneration, cryopreservation, micropropagation, protoplasts, transgenics	Zhang et al. 2007; Boine et al. 2008; Haggman et al. 2008; Kanwar et al. 2008; Shen et al. 2008; Kanwar et al. 2009
Salix	Cryopreservation	Towill and Ellis 2008
Ulmus	Embryogenesis, protoplasts	Conde and Santos 2006

modified Gresshoff and Doy medium (Gresshoff and Doy 1972) containing 2.22 μM BA for shoot initiation and 0.44–0.89 μM BA for multiplication, with various concentrations (14.76 μM to 14.76 mM) and exposure times (1–2 min to 5–7 d) of IBA for rooting. Goncalves et al. (2008) measured the endogenous levels of indole-3-acetic acid (IAA), IBA, and indole-3-acetylaspartic acid during the first 8 d of *in vitro* rooting of a hybrid adult chestnut, and they concluded that IAA was the signal responsible for root induction driven by exogenous application of IBA. *In vitro* propagation of *Fraxinus excelsior* L. (European ash), an important timber species, using epicotyl explants cultured on WPM supplemented with 0.05 μM IBA, 13.32 or 17.76 μM BA, or 0.45 or 2.27 μM TDZ has been reported (Mitras et al. 2009). The highest percent of adventitious root formation (97%) was induced on shoots using WPM containing 2.46 μM IBA plus 2.69 μM NAA for 14 days. Black walnut (*Juglans nigra* L.), a commercially valuable timber species, has been micropropagated on semi-solid MS and Driver and Kuniyuki walnut (DKW; Driver and Kuniyuki 1984) medium and in a liquid DKW medium supplemented with various cytokinins and auxin with good shoot proliferation (Roschke and Pijut 2007; Bosela and Michler 2008). Bosela and Michler (2008) reported that the cytokinin type and concentrations required for axillary proliferation had adverse effects on the morphology of the shoots. Advances in the micropropagation of English or

Persian walnut (*Juglans regia* L.) hybrids and cultivars continues to be successful (Amiri 2006; Fatima et al. 2006; McGranahan and Leslie 2006; Britton et al. 2007; Rios Leal et al. 2007; Hackett et al. 2009; Vahdati et al. 2009a, b). English walnut has been cultivated for nut production for thousands of yr, and *in vitro* biotechnology efforts have been under way for more than a decade. While some standard micropropagation protocols have been developed, it has been reported that culture media formulations and plant growth regulators often still need to be optimized for specific cultivars and clones (Britton et al. 2007). Adventitious rooting of *in vitro* propagated *J. regia* selections also continues to progress (Caboni and Damiano 2006; Leslie et al. 2006; Pei et al. 2007; Sharifan et al. 2009; Vahdati et al. 2009a, b). As with micropropagation, adventitious rooting can be genotype or cultivar dependent. Oaks (*Quercus* spp.) are a major source of timber and important mast producers for many organisms (Johnson et al. 2002). Regeneration of oaks is gradually deteriorating as a result of extensive harvesting, unavailability of seeds every yr, and predation. Micropropagation of several important North American oak species continues to develop (Vengadesan and Pijut 2009a; Vieitez et al. 2009). Northern red oak (*Quercus rubra* L.) cotyledonary node explants (73.3%) regenerated shoots when cultured on WPM containing 4.4 μM BA, 0.29 μM gibberellic acid, and 500 mg L^{-1} casein hydrolysate; with rooting of elongated shoots achieved on WPM with 4.9 μM IBA (Vengadesan and Pijut 2009a). Vieitez et al. (2009) stated that the addition of 3 mg L^{-1} $AgNO_3$ to WPM reduced shoot tip necrosis and early senescence of leaves of northern red oak shoot cultures, and rooting was achieved in medium containing 123 μM IBA for 48 h. *Quercus alba* L. (white oak) and *Quercus bicolor* Willd. (swamp white oak) micropropagation has also been achieved, but marked differences occurred in explants from different genotypes (Vieitez et al. 2009). *In vitro* propagation of brown oak (*Quercus semecarpifolia* Sm.), an important Indian Himalayan forest tree species, has been achieved using a part of the petiolar tube containing a primary shoot as the initial explant (Tamta et al. 2008). *Quercus euboica* Pap., a rare and endangered oak species endemic to the Greek Island Euboia (middle eastern Greece), was micropropagated from young seedlings or adult plants using WPM supplemented with 4.44 μM BA, and rooting was achieved in medium containing 9.84 μM IBA (Kartsonas and Papafotiou 2009). Adventitious rooting of micropropagated cork oak (*Quercus suber* L.), a Mediterranean species harvested for cork production, has been investigated at the molecular level (Neves et al. 2006). There was a tendency for higher expression of a B-type cyclin in auxin-induced cork oak shoots associated with the first cell divisions leading to root primordia (Neves et al. 2006). Flow cytometry has been utilized to determine genetic fidelity of *in vitro*-propagated cork oak (Santos et al. 2007). Micropropagation of several other oak species [*Quercus cerris* L. (European turkey oak), *Q. rubra*, *Quercus robur* L. (English oak), and *Quercus virgiliana* Ten. (Italian pubescent oak)] using dormant buds has also been reported (Ostrolucka et al. 2007). Improvements to the micropropagation of black locust (*Robinia pseudoacacia* L.) for germ plasm conservation and investigations of the plant-associated bacteria in tissue cultures that can be a serious problem with propagation have been described (Zhang et al. 2007; Boine et al. 2008). Substantial progress has been made in the micropropagation of mature temperate hardwood tree species, but further research is needed to continue to conserve elite germ plasm.

Adventitious shoot regeneration. Adventitious shoot regeneration from unusual points of origin (Schaeffer 1990) is usually not desirable for "clonal" propagation of a species because of the possibility of somaclonal variation. However, these protocols are useful in the genetic improvement and conservation of a species (see Table 1).

Four different culture mediums (modified MS with various concentrations and types of plant growth regulators) were utilized for callus induction, callus proliferation, shoot differentiation, and rooting of European white birch (Haggman et al. 2007). Significant differences in the effects of genotype, explant, cytokinin, and dark treatment on shoot regeneration from both leaf and petiole tissue of two birch species was also reported (Magnusson et al. 2009). An adventitious shoot regeneration and rooting protocol was developed for green ash, an important North American hardwood threatened by the emerald ash borer (Du and Pijut 2008). The best regeneration (76%) from green ash hypocotyls was on MS medium supplemented with 13.3 μM BA plus 4.5 μM TDZ. Plants of Formosa sweetgum (*Liquidambar formosana* L.), a non-native species planted in the North American urban environment as a large shade tree, were regenerated (73–90%) from leaf explants of five genotypes on WPM supplemented with 1.14 μM TDZ and 0.27 μM NAA (Xu et al. 2007). Leaf explants from *in vitro*-grown plants of *Platanus occidentalis* L. (American sycamore), a species grown in short-rotation plantations primarily for pulp and rough lumber, were used successfully to regenerate shoots on WPM containing 22.2 μM BA and 0.49 μM IBA (Sun et al. 2009). Liu and Pijut (2008) reported adventitious shoot regeneration and rooting of *Prunus serotina* Ehrh. (black cherry), a hardwood species valued for its wood, from two mature genotypes using *in vitro* leaf explants cultured on WPM with TDZ and NAA. Percent regeneration was genotype dependent, and silver thiosulfate at 60 or 80 μM increased the percent regeneration of the mature genotypes. Regeneration of plants from cell suspension culture, and callus- and mesophyll-derived protoplasts of black locust, a

nitrogen-fixing multipurpose tree used in intensive forest improvement as well as land reclamation, was developed using various techniques, culture medium and plant growth regulators (Kanwar et al. 2008, 2009). *In vitro* leaves of *Ulmus minor* Mill. (English elm), a species threatened by Dutch elm disease, were used successfully for obtaining high yields of protoplasts, and micro-calli transferred to solid medium developed into embryogenic callus (Conde and Santos 2006).

Somatic embryogenesis. Somatic embryogenesis from vegetative or non-gametic cells (Schaeffer 1990) of important temperate hardwood species can provide a means for mass propagation of selected, endangered, hybrid or transgenic (disease or pest resistant) species (Table 1).

An improved germination and plant recovery technique was reported for European chestnut (Corredoira et al. 2008). Fast desiccation (2 h) of mature somatic embryos, previously exposed to cold treatment for 2 mo, enhanced plant recovery. The addition of 0.44 μM BA, 200–438 mg L^{-1} glutamine, and 0.49 μM IBA improved plant quality. Somatic embryogenesis of *Fagus grandifolia* Ehrh. (American beech), an important wood and mast species, from five putatively resistant (to beech scale) mature trees, was initiated (Pond 2008). Somatic embryos were induced from zygotic embryos and young leaves from mature trees, but germinated somatic embryos did not survive transfer to soil. Advances in somatic embryo maturation and germination of Persian walnut, a commercial timber and nut tree species, continues to be reported (Vahdati et al. 2006, 2008; Bahrami Sirmandi and Vahdati 2009; Baya et al. 2009). Different genotypes were used in these studies, and the effects of abscisic acid, carbohydrate source (sucrose or maltose), or osmoticum (polyethylene glycol) on somatic embryo maturation, germination and conversion into plants were investigated. Addition of 0.01–5 μM TDZ in the germination medium for somatic embryos of *Q. robur* induced multiple shoot formation, and adventitious bud regeneration (69%) was induced on the cotyledons of somatic embryos treated with 0.5 μM TDZ (Martinez et al. 2008). Embryogenic callus was induced on immature cotyledon explants of northern red oak using MS medium containing 0.45 μM 2, 4-dichlorophenoxyacetic acid (2,4-D) (Vengadesan and Pijut 2009b). Higher numbers of globular-, heart-, torpedo-, and cotyledon-stage somatic embryos were then obtained after culture on medium devoid of 2,4-D, with germination and development of plants after desiccation and cold storage. *In vitro* studies on *Q. suber* embryogenesis and diploidization of anther-derived embryos (for breeding purposes) continues to advance the improvement, conservation, and production of this important forest and agro-forestry species (high-quality cork; acorns for feed for cattle and pig farming; Celestino et al. 2007, 2009; Pintos et al. 2007, 2008; Gomez et al. 2009). Anther-derived haploid embryos from six genotypes were treated with three different anti-mitotic agents, and 0.01 mM oryzalin (for 48 h) produced approximately 50% diploid embryos (Pintos et al. 2007). Pintos et al. (2008) encapsulated somatic embryos of cork oak for the production and storage (4°C for 2 mo without significant loss) of synthetic seeds, and monitored the growth with a digital image system. Celestino et al. (2007, 2009) and Pintos et al. (2009) reported on field trials of cork oak plants regenerated via somatic embryogenesis, and they concluded that the regenerated plants showed a cork quality trait similar to that of the parent tree. Proteomic analysis of haploid and diploid embryos of cork oak identified 17 differentially expressed proteins; of which some were involved in the synthesis of cork chemical components (Gomez et al. 2009). This research shows promise for screening for biomarkers related to cork quality.

Cryopreservation. Cryopreservation, the ultralow temperature (−196°C) storage of cells, tissue, embryos, or seeds (Benson 2008), is a viable secondary storage form that can be considered as a secure backup to living collections (Reed 2008). Cryopreservation of certain genotypes involves multiple steps to be successful (choice of material, pretreatment, freezing, storage, thawing, and post-treatment handling) when working with hardwood tree species. Cryopreservation for conservation purposes allows for storage of valuable seed (some recalcitrant seeds), pollen, shoot tips, meristems, axillary and dormant buds, embryonic axes, zygotic or somatic embryos, genetically modified lines, callus, or cell cultures depending on the species. A recent book includes the theory, descriptions, and procedures most commonly used in the cryopreservation of some important species (Reed 2008; Table 1).

Cryopreservation of buds from *B. pendula* trees having specific leaf color variegation (golden veined or white flecked) was reported, but the 1- and 2-yr-old progenies failed to express this leaf variegation, suggesting that this trait was genetically determined and regulated by different epigenetic factors (Ryynanen and Aronen 2007). *In vitro* shoot tips from an elite *B. pendula* clone were cryopreserved by adaptation of the plant vitrification solution 2 protocol (Meier-Dinkel 2007). The highest shoot formation reported (60%) after freezing in liquid nitrogen was achieved after 3 wk of cold hardening and 3 d of preculture on WPM with 0.8 M glycerol. Haggman et al. (2008) summarized the conservation strategies for deciduous forest tree resources and described the cryopreservation protocols used for silver birch (European white birch) and black locust. Vieitez et al. (2007) described the steps involved in cryopreservation and re-growth of European chestnut shoot tips. Transgenic European chestnut somatic

embryos were successfully cryopreserved using a vitrification-based procedure, and plants recovered after maturation and germination conditions (Corredoira et al. 2007). Lambardi et al. (2008) summarized the protocol used for vitrification of embryogenic cultures of chestnut, common oak, and cork oak. Towill and Ellis (2008) summarized the protocols for cryopreservation of dormant buds taken from silver birch and willow. Roach et al. (2008) indicated that a complex stress interaction is induced (an oxidative burst of superoxide) in embryogenic axes of sweet chestnut between excision and subsequent drying in preparation for cryopreservation. Chmielarz (2009) reported that a 2-yr seed storage period of dormant European ash (Polish provenance) in liquid nitrogen after desiccation to the critical (safe range) water content of 0.06–0.24 g g^{-1} resulted in no decrease in germination after thawing. Stem sections with dormant buds of three ash species (*F. pennsylvanica*, *Fraxinus mandshurica* Rupr. [Manchurian ash], and *Fraxinus chinensis* Roxb. [Chinese ash]) were successfully cryopreserved when desiccated to 30% (*v*/*v*) moisture content, cooled and immersed in liquid nitrogen vapor; grafting of buds onto rootstocks after cryopreservation resulted in 34–100% recovery (Volk et al. 2009). Cryostorage of embryogenic lines derived from mature English oak trees resulted in 57–92% embryo recovery, with no genetic instability observed in regenerated plants after random amplified polymorphic DNA analysis (Sanchez et al. 2008). Cryopreservation of cork oak somatic embryos resulted in 90% survival, and little to no genetic instability was observed after assessment by flow cytometry, amplified fragment length polymorphisms (AFLPs) and simple sequence repeats (SSRs; Fernandes et al. 2008).

Marker Technologies in Conservation and Tree Improvement

A DNA marker refers to the variance within a specific region of DNA among individuals of a species. DNA markers can be used early in tree growth and development to predict dissimilar genetic backgrounds and to determine which traits a particular individual is carrying by examining these small segments. Markers can discern between single or multi-locus modifications as well as dominant or co-dominant alterations in a single individual. When applied to the conservation and breeding of fine hardwoods, many diverse DNA marker system types have been utilized. DNA markers allow for more accurate determination of the region of origin for a particular tree and detection of specific gene flow events. The many DNA marker techniques are similar in that these can be used even where there is a single nucleotide change in a gene or tandem DNA repeat. Unlike morphological markers, these changes are not apparent in the phenotype of the individual and are often insignificant in its physiological development. This review examines several DNA marker techniques with reference to improvements made in maintaining diversity within hardwood forests.

Isozymes. Historically, the most common biochemical markers have been isozymes. The term isozyme, sometimes referred to as allozyme, refers to alternative forms of an enzyme that were differentially coded by alleles found at the same genetic locus. Isozymes are a robust and inexpensive method of screening in many fields of biology. Co-dominant isozyme markers allow for distinguishing heterozygotes from homozygotes within a sample. The main drawback is that it may underestimate the degree of genetic variation within an experimental paradigm because of cryptic alleles. In a variation of this technique, isoelectric focusing, the isozymes are focused via gel electrophoresis to a point of equal charge and are then migrated according to size.

Isozymes have been used in forest tree conservation research since the early 1970s. The use of isozymes to study genetic diversity and outcrossing rates and relatedness within wild pecan (*Carya illinoinensis* Koch.) populations represents one of the earliest uses for these genetic markers in a fine hardwood species (Rüter et al. 1999, 2000). However, few other studies have been conducted using this technique alone.

Restriction fragment length polymorphism. Restriction fragment length polymorphism (RFLP) is a DNA-based technique modeled after Southern blotting (Harvey and Soundy 2005). This procedure involves generation of a genomic library so that a particular restriction enzyme digested sample may be screened against a group of samples in order to determine mutations at the enzyme cleavage site that resulted in insertions or deletions in the target DNA. This method reveals more genetic variation than isozyme procedures while maintaining high reproducibility.

Burg et al. (1993) used petunia (*Petunia hybrida*) chloroplast DNA (cpDNA), which was highly conserved, to screen for polymorphisms within a small population of pedunculate oaks (*Q. robur*) and sessile oaks (*Quercus petraea* [Matt.] Liebli.). The goal was to trace the inheritance of highly conserved sequences to determine evolutionary distances between populations. Abuín et al. (2006) reported on the development of RFLP-polymerase chain reaction (PCR) to obtain *Juglans* spp. specific markers. Five *Juglans* spp. (*J. regia*, *J. nigra*, *Juglans major* (Torr.) A. Heller, *Juglans hindsii* (Jeps.) Jeps. Ex R. E.Sm., and *Juglans australis* Grise.) and the maternal relationships between the taxa were examined. Magni et al.

(2005) examined *Q. rubra* populations using RFLP, and while unable to determine the extent of hybridization, hypothesized that additional study of other North American *Quercus* spp. may offer an explanation as to why many *Quercus* spp. of similar geographic origin are structurally dissimilar. Petit et al. (2003) examined European *Quercus* spp. and compiled the resultant haplotype information for *Q. robur*, *Q. petraea*, *Quercus pubescens* Willd., and *Quercus pyrenaica* Willd. Birchenko et al. (2009) with *Q. rubra* indicated a loss of haplotype diversity along the northern edge of the distribution range providing a possible explanation for the morphological differences. Because of advances in genetics and analysis of quantitative traits, RFLPs are not commonly used.

Random amplified polymorphic DNA-PCR. Random amplified polymorphic DNA (RAPD)-PCR uses PCR-based amplification of anonymous genome regions to examine polymorphisms within the DNA sequence. By synthesizing fragments of DNA between two identical primers of about ten base pairs in length, it is possible to sample many sites on the genome. Because of the small amounts of DNA required, use of RAPD-PCR has been quite popular. Since its inception, extensive variation has been detected in all tested organisms (Rafalski et al. 1991); however, it has not proven to be robust against slight changes in environment or PCR conditions introducing variation. Also, dominant markers were the only ones identified, although some may be co-dominant. When combined with RFLP, RAPD-PCR may be used to generate a DNA fingerprint of the individual tree.

Howland et al. (1995) used RAPD-PCR to investigate the natural genetic variations between silver birch (*B. pendula*) and downy birch (*Betula pubescens* Ehrh.) on the basis of ploidy. The two species were indistinguishable on the basis of morphology or molecular characteristics, except in East Anglian populations, as a result of the inherent high degree of polymorphisms common within *Betula* spp. These results indicate that a considerable amount of crossing occurs between the two species outside the East Anglian region.

Amplified fragment length polymorphisms. Amplified fragment length polymorphisms (AFLPs) have been among the most popular methods to identify individual trees. An amalgam of RFLP and PCR, AFLPs were frequently used for the early construction of genetic plant maps (Vuylsteke et al. 2006). Proven to be more robust than RAPD-PCR, AFLPs can be subject to difficulties in replication of results. Despite this, the use of AFLPs in forest tree research has continued to grow over the last decade. Cervera et al. (2000) used this technique to analyze data for a number of tree species and were able to generate reproducible AFLP fingerprints for 15 clones of both pedunculate oak and sessile oak. This technique has been used in both the identification of individual members and in the determination of gene flow between populations of *Acer* (Van Huylenbroeck et al. 2004; Bless et al. 2006), *Alnus* (Huh and Huh 2001), *Betula* (Kulju et al. 2004; Schenk et al. 2008), *Carya* (Vendrame et al. 2000; Beedanagari and Conner 2004), *Celtis* (Whittemore 2005; Whittemore and Townsend 2007), *Fagus* (Jump and Peñuelas 2007), *Prunus* (Van Huylenbroeck et al. 2004), *Quercus* (Coart et al. 2002; Hipp and Weber 2008), *Robinia* (Huo et al. 2009), *Tilia* (Van Huylenbroeck et al. 2004), and *Ulmus* (Whiteley et al. 2003) within familial groups. AFLP analysis has been used to determine the levels of genetic and structural diversity within natural and artificial populations (Huh and Huh 2001; Coart et al. 2002). Clark et al. (2001) were able to further classify *Castanea* spp. using AFLP markers from the *Castanea mollissima* Blume.–*C. dentata* map in comparison with those from the *C. sativa* map to uncover differences in geographic origin within a group of trees. Feng et al. (2007) report AFLPs allow for selection against poor nut attributes in *Castanea* spp.

Differentiation between *Quercus* spp. remains challenging because of highly variable traits in conjunction with polymorphisms and hybridization throughout time (Ishida and Kimura 2003). Ishida and Kimura (2003) noted that sibling pairs of *Quercus* hybrids were more easily distinguished by morphological traits rather than DNA marker examination. Pooler and Townsend (2005) indicated success with AFLP in determining geographic origins and genetic distances between selections of *Ulmus laevis* Palli. and *Ulmus americana* clones. Whittemore and Townsend (2007) used AFLP to assess parentage within controlled *Celtis occidentalis* populations, as juvenile and adult morphology differ drastically. AFLPs have also been used in the evaluation of trees derived from somatic embryogenesis, such as *Q. petraea*, *Q. robur*, and *Q. suber* (Wilhelm 2000; Hornero et al. 2001). Hornero et al. (2001) indicated that AFLP analysis uncovered twice as many markers as RAPD.

Microsatellites, SSRs, and inter-simple sequence repeats. Microsatellites (also known as SSRs) and inter-simple sequence repeats (ISSRs) refer to short tandem repeats often found in the 5-prime untranslated regions of eukaryotic genomes. The number of repeats within a unit is highly variable, a characteristic that makes this a highly polymorphic region. ISSR is similar to RAPD; however, ISSR primers are longer than RAPD primers, providing increased stringency and greater reproducibility.

Potter et al. (2002) established phylogenetic relationships among sampled black walnut (*J. nigra*) and Persian walnut (*J. regia*) cultivars with great accuracy using ISSRs.

Pollegioni et al. (2009) resolved phylogenetic relationships among hybridogenic *Juglans intermedia* (*J. nigra* x *J.*

regia) natural mixed populations using microsatellites without the aid of phenological or morphological data. The first report of its kind, Pollegioni et al. (2009) used ten microsatellite loci, pollen analysis, and a small sample of parent trees to identify 198 diploid progeny in addition to pinpointing the most reproductively successful male parent trees. King and Ferris (2000) incorporated ISSRs and cpDNAs for sympatric distribution studies of *Alnus cordata* Lois. and *Alnus glutinosa* (L.) Gaertn., and noted that cpDNA variation was geographic in nature, while ISSR data was taxonomic; indicating that breeding and conservation efforts must take into account these differences. Microsatellite markers were first generated for *Q. rubra* by Aldrich et al. (2002, 2003), while Romero-Severson et al. (2003) reported that RFLPs helped uncover spatial scales and sample sizes necessary for accurate determination of haplotype frequency. The first use of ISSR and SSR in *Q. suber* allowed differentiation between individual elite trees based on three microsatellite loci originally used for *Q. petraea* (López-Aljorna et al. 2007). Aldrich et al. (2005) examined regeneration decline within *Quercus* old-growth forests in Indiana using microsatellites to determine whether understory composition factored in the resultant transfer of genes throughout generations. Buiteveld et al. (2007) examined whether forest growth management practices played a role in mating and genetic diversity within *Fagus sylvatica* forests, while Kraj and Sztorc (2009) examined *F. sylvatica* early, intermediate and late phenological forms. Also in *F. sylvatica*, Jump and Peñuelas (2007) used AFLP in the appropriation of spatial genetic structure. Guarino et al. (2008) made efforts to survey genetic variability among various spatial scales within *Acer* assemblages. Mylett et al. (2007) reported that RAPDs were used to indicate genetic variability between individual *Tilia cordata* Mill. clusters within the same woodland and surrounding areas. In *F. excelsior* (Þvingila et al. 2005) and in *B. pendula* (Jiang et al. 2007), it was noted that the unique RAPD phenotype, the basis for individual tree identification, indicated that RAPD markers can indeed confirm origin of a given forest tree.

Many DNA marker techniques have been implemented to measure genetic stability of *in vitro*-propagated plant lines of *Platanus* x *acerifolia*, a hybrid of *Platanus orientalis* x *P. occidentalis* (Huang et al. 2009). Huang et al. (2009) remarked that use of ISSR has generated the most promising results because of the superior sensitivity of the method. Other techniques, such as RFLPs and RAPDs used in earlier studies involving use of *Platanus* spp. and hybrids, successfully concluded analysis of plant parentage as well (Besnard et al. 2002), implying that many methods may be used successfully. While systems have been developed for the determination of parentage of *in vitro* plants, regeneration of these plants from leaf tissue was a more difficult task. A simple, efficient, and genetically stable method for *P. occidentalis* was recently presented using RAPDs by Sun et al. (2009), who suggested this system may be amenable to other *Platanus* spp.

Fine hardwood microsatellite research has also centered on sustainability with seed and pollen dispersal. Examinations of wind-pollinated tree species, such as *F. excelsior* (Morand-Prieur et al. 2003; Heuertz et al. 2003, 2004; Bacles and Ennos 2008), *F. sylvatica* (Sander et al. 2000; Vornam et al. 2004; Scalfi et al. 2004, 2005; Jump and Peñuelas 2006), and *Quercus* spp. (Valbuena-Carabaña et al. 2005; Austerlitz et al. 2007; Pluess et al. 2009), using paternity information generated from microsatellite data, note that pollen-associated restricted gene flow may contribute to loss of genetic diversity within forests. However, the methodologies and sample sizes used were important considerations when evaluating statistical results (Jump and Peñuelas 2007).

Attempts to detect patterns of genetic variation among hardwood tree species along vertical and horizontal mountain gradients has been an ongoing investigation using a myriad of genetic methods and genotypes. AFLPs were implemented for *F. sylvatica* studies (Jump and Peñuelas 2007) while SSRs were used to study *B. pubescens* (Truong et al. 2007) populations occupying the same distribution range as *B. pendula*. Ohsawa et al. (2007a, b) and Ohsawa and Ide (2008) used SSRs, isozymes, and RAPD analyses to analyze variation among *Quercus crispula* and *Quercus serrata* at different altitudes. Ross-Davis et al. (2008a) used microsatellites to clarify the role that demographics played in the maintenance of allelic diversity within *Juglans cinerea* populations. Ross-Davis et al. (2008b) used RAPDs to differentiate between pure *J. cinerea* and hybrid individuals. Pollegioni et al. (2006) compared ISSR, RAPD, and SSR methodologies in an effort to distinguish between parental species and the resultant progeny among *J. regia* and *J. nigra* natural and artificial hybrids. It was apparent that a number of advanced techniques and methodologies must be invoked to provide the most accurate information pertaining to hardwood forests.

Pairon et al. (2006a) modeled the dispersal distances of *P. serotina* using microsatellites and compared these results to those of Jordano and Godoy (2001) with *Prunus mahaleb* L. Pairon et al. (2006a, b) applied microsatellite marker technology to assign a maternal parent to its dispersed seed and noted that dispersal distances were quite short, indicating that many seeds fell close to the parent trees. Using this newly attained information and microsatellites, Pairon et al. (2008) sought to identify genome-specific markers for *P. serotina* that would aid in the study of progeny inheritance patterns in North American and European cultivars. Downey and Iezzoni (2000) ascertained that SSRs from several *Prunus* spp. could also be used for *P. serotina*, a conclusion seconded with experimentation by

Wünsch and Hormaza (2007). The use of *P. serotina*, *Q. robur*, and *Q. petraea* cpDNA has shown that significant diversity can be seen even in the presence of low sequence polymorphisms (Petit et al. 2002; Petitpierre et al. 2009).

Efforts to improve the quality of tree nut crops have been more pronounced for temperate species than tropical ones, and it has been speculated that the increased numbers of breeding programs and funding opportunities available have contributed to the continued improvements in tree nut genetics (Mehlenbacher 2003). Used in mapping studies with nut crops and fruit trees, microsatellite analysis has been successfully reported for *C. sativa* (Buck et al. 2003; Marinoni et al. 2003; Sisco et al. 2005; Tanaka et al. 2005; Batista et al. 2008), *C. illinoinensis* (Beedanagari et al. 2005), *F. sylvatica* (Scalfi et al. 2004), *Prunus avium* (Clarke and Tobutt 2003; Vaughan and Russell 2004; Dondini et al. 2007; Dirlewanger et al. 2008; Olmstead et al. 2008), *Q. robur* (Barreneche et al. 2004; Parelle et al. 2007), *R. pseudoacacia* (Mishima et al. 2009), and *J. nigra* (Victory et al. 2006) species.

To maintain diversity within fine hardwood forests, additional research with respect to management and conservation must be initiated as indicated with *C. sativa* (Wang et al. 2008b), *F. sylvatica* (Buiteveld et al. 2007), *F. excelsior* (Heuertz et al. 2004; Harbourne et al. 2005), *J. regia* (Aradhya et al. 2007; McGranahan 2007), and *Ulmus spp.* (*Ulmus glabra* Huds., *U. laevis*, and *U. minor*) (Goodall-Copestake et al. 2005). Additionally, care must be taken during the assemblage of parentage and mating data, as the use of different genetic approaches yields different information (Hardy et al. 2006). It is entirely possible that future studies in genetic techniques will reveal discrepancies between biogeographical evidence and fossil records (Aradhya et al. 2007). Studies of *Liriodendron tulipifera* (Xu et al. 2006; Yao et al. 2008), *Acer opalus* Mill. (Gleiser et al. 2008a, b; Segarra-Moragues et al. 2008), *F. sylvatica* (Jump and Peñuelas 2007), *F. excelsior*, and *Fraxinus angustifolia* (Morand-Prieur et al. 2003), *Quercus virginiana*, and *Quercus geminata* (Cavender-Bares and Pahlich 2009), *Q. robur*, *Q. petraea*, *Q. pubescens*, and *Q. pyrenaica* (Lepais et al. 2009) have shown that the use of microsatellites, SSRs, and ISSRs were able to generate parentage data within forest ecosystems. With genealogy information completed, researchers will be better able to determine which conservation and management practices will provide the most benefit to maintaining allelic diversity within stands.

Sequence characterized amplified regions. Sequence characterized amplified regions (SCARs) are DNA fragments that have been PCR amplified using specific primers designed from nucleotide sequences determined from RAPD-PCR. This method has greater specificity than RAPD-PCR by using longer PCR primers. AFLPs can be converted to SCARs by using the same basic method as for RAPD-PCR. To improve the economic value of *B. platyphylla*, Wang et al. (2008a) used RAPD and SCAR to analyze birch trees with the greatest fiber length for use in the selective breeding process, while Grauke et al. (2003) explored microsatellites and SSRs for improved scoring and resolution within pecan.

Additional Marker Techniques

Diversity arrays technology. Diversity arrays technology uses gene chips or microarrays to examine a multitude of loci at one time. To decrease complexity, cDNA or RNA is hybridized to a slide. The slide is probed with a fluorescently labeled probe and then scanned for polymorphisms. This technique is robust and high-throughput while eliminating the need for either agarose or polyacrylamide gels. Barros et al. (2009) provided the most up-to-date information on implementation of this system in forest tree research and other areas of plant genetics and biotechnology.

Single nucleotide polymorphisms. Single nucleotide polymorphisms (SNPs) were not used in genotyping until the advent of rapid sequencing technologies (Brookes 1999). Zhang and Zhang (2005) discussed advances in the field of SNPs and provided a compilation of published data with respect to forest trees. While their review did not cover any North American fine hardwoods, their final outlook indicated that the SNP technologies were apt to be used in the future for all tree species.

Sequence tagged sites. First adapted in 1989 by Olson et al., sequence tagged sites (STSs) tend to be co-dominant and allow for amplification of short smaller scale sequences amongst a myriad of genetic sequence, thus facilitating recognition between heterozygotes and homozygotes. Few, if any, reviews on the use of STSs in a hardwood species have been published in recent yr.

Additional information concerning the design and implementation of the many techniques or any of the aforementioned tree species are available at several website locations, such as http://www.diversityarrays.com http://www.ncbi.nlm.nih.gov/projects/genome/probe/doc/Technologies.shtml http://dendrome.ucdavis.edu/

Genomic Tools and Genetic Engineering in Hardwood Tree Improvement

Quantitative trait loci mapping. Use of marker techniques allow for the mapping of economically important traits and

subsequent production of linkage maps important for understanding genes controlling complex traits. There are several mapping procedures used in forestry with quantitative trait loci (QTL) mapping being discussed here. QTL refers to the chromosomal region thought to contain genes responsible for variations seen in a trait (Mauricio 2001).

Studies indicate the usefulness of this specialized technique in the identification of disease-resistance, growth, quality and yield traits (Groover et al. 1994; Grattapaglia et al. 1995; Plomion et al. 1996; Conner et al. 1998), but it may have limited advantages in fine hardwood species with the most difficult aspect of QTL mapping being resolution. Pijut et al. (2007) describe at length these difficulties and illustrate information that can be provided using this technique for genetic improvement of fine hardwoods. Developed in stone fruit trees and nut crops (Dondini et al. 2007; Pierantoni et al. 2007; Dirlewanger et al. 2008), QTL in conjunction with microsatellite analysis has been successfully reported for *F. sylvatica* (Scalfi et al. 2004) and *Q. robur* (Parelle et al. 2007). Scalfi et al. (2004) reported the first linkage map for *Fagus* to examine traits such as leaf size and shape, and carbon isotope discrimination ($\Delta^{13}C$), while Parelle et al. (2007) reported on waterlogging tolerance traits among European oak. Brendel et al. (2008) described QTLs controlling water use efficiency and related traits in (*Q. robur*). In a comparison of QTL positions between *Q. robur* and *C. sativa*, Casasoli et al. (2006) described the occurrence of orthologous microsatellites and ESTs.

Linkage maps enable the identification of regions of genes that control a specific subset of adaptive traits. Once a particular region of interest has been identified, marker-assisted selection can help to eliminate sub-par seedling and juvenile trees while allowing the strongest trees with the most desired traits to breed (Thompson and Grauke 2000; Beedanagari and Conner 2004; Dvorak et al. 2007; Dirlewanger et al. 2008). QTL analysis has been used to isolate a number of crucial forest tree traits such as bud phenology, nut quality and quantity, growth and yield, water use efficiency, and wood quality (Jermstad et al. 2001; Casasoli et al. 2004; Scalfi et al. 2004). Gailing et al. (2008) used QTL analysis to confirm the contribution of genetic variation to stomatal density within a single *Q. robur* family. Gao and Jiang (2009) constructed a genetic linkage map for *B. pendula* based on AFLP markers for use in QTL analysis. It was the opinion of these authors that the continued use of QTL analysis will allow forest tree plantation owners, breeders, and smaller-scale tree farmers to employ these data to selectively breed high-quality hardwoods.

Genomics in hardwood biotechnology. The integration of genome information into fine hardwood studies will serve as the basis for understanding the tools used in functional genomics research; namely, genetic mapping and QTL analysis. Worldwide, research programs dedicated to completing genome sequences for fine hardwoods have received acclaim. In 2007, Europe renewed its dedication to "maintain, conserve, restore, and enhance the biological diversity of forests, including the genetic resources, through sustainable forest management…" as part of the Warsaw Declaration. The European Forest Genetic Resources Programme (EUFORGEN 1994) currently facilitates availability of many forest genetic resources in Europe. In France, FOR*EST*, an initiative to catalogue *Quercus* EST genomic sequences, is working alongside the Biodiversity Genes, and Communities and the Institut National de la Recherche Agronomique (INRA 2010) joint research Unite Mixte de Recherche Ecologie et Ecophysiologie Forestiere to examine adaptation responses of oak trees to climatic changes using current genetic diversity information. Similarly in Portugal, SuberGene (Paiva et al. 2010), a collaboration between France and Portugal, compiles and studies *Q. suber* ESTs to gain insight into cork formation and variability. Initiation of The Floral Genome Project (2010) in the USA, an EST database for genes and gene families expressed in early flower development, has provided the first resource for *L. tulipifera* functional and comparative genomic sequence information (Liang et al. 2008). The Fagaceae Genome project (Fagaceae Genomics Web 2006), funded by the National Science Foundation, has spurred development of EST databases for five fine hardwood species; American chestnut (*C. dentata*), Chinese chestnut (*C. mollissima*), northern red oak (*Q. rubra*), white oak (*Q. alba*), and American beech (*F. grandifolia*). Nearing its completion, this project has already provided critical information for new discoveries in the field of conservation research (Barakat et al. 2009; Sisco et al. 2009). In addition, other studies focusing on hardwood species have been developed and implemented in recent years, including the completion of the peach (*Prunus persica*) genome database (Jung et al. 2007), pyrosequencing of the *P. occidentalis* plastid genome (Moore et al. 2006), organization of a microsatellite cohort for *Fraxinus* spp. (Lefort et al. 2000; Harbourne et al. 2005), and the phylogenetic analysis of genome information for *Juglans* spp. (Aradhya et al. 2007) and *Liquidambar styraciflua* (Morris et al. 2008). Additional genome information, sequences for coniferous species and *Populus trichocarpa* (Western poplar) and germplasm information is available at the Dendrome (2010); Phytozome (2010), and the National Clonal Germplasm Repository for Fruit and Nut Crops (NCGR 2010) websites. A summary of genetic techniques used in fine hardwood tree biotechnology research is presented in Table 2.

Genetic transformation. Tissue culture and genetic transformation techniques have allowed for incorporation of

Table 2. Summary of genetic techniques used in fine hardwood tree biotechnology research

Genus	Technique(s)	References
Acer	AFLP, microsatellites, RAPD	Bless et al. 2006; Gleiser et al. 2008a, b; Guarino et al. 2008; Segarra-Moragues et al. 2008; Van Huylenbroeck et al. 2004
Alnus	AFLP, cpDNA, ISSR, RAPD, RFLP, SCAR	Huh and Huh 2001; King and Ferris 2000
Betula	AFLP, microsatellites, QTL, RAPD, SCAR, SSR	Gao and Jiang 2009; Howland et al. 1995; Jiang et al. 2007; Kulju et al. 2004; Schenk et al. 2008; Truong et al. 2007; Wang et al. 2008a; Wang et al. 2008b
Carya	AFLP, isozymes, microsatellites, SSR	Beedanagari and Conner 2004; Beedanagari et al. 2005; Grauke et al. 2003; Röter et al. 1999; 2000; Vendrame et al. 2000
Castanea	AFLP, isozymes, ISSRs, microsatellites, QTL, RFLP, RAPD, SSR	Barakat et al. 2009; Batista et al. 2008; Buck et al. 2003; Casasoli et al. 2004; Casasoli et al. 2006; Clark et al. 2001; Feng et al. 2007; Marinoni et al. 2003; Sisco et al. 2005, 2009; Tanaka et al. 2005; Wang et al. 2008b
Celtis	AFLP	Whittemore 2005; Whittemore and Townsend 2007
Fagus	AFLP, microsatellites, QTL, RAPD	Buiteveld et al. 2007; Jump et al. 2006; Jump and Peñuelas 2007; Kraj and Sztorc 2009; Sander et al. 2000; Scalfi et al. 2004; Scalfi et al. 2005; Sisco et al. 2009; Vornam et al. 2004
Fraxinus	Microsatellites, RAPD, RFLP	Bacles and Ennos 2008; Harbourne et al. 2005; Heuertz et al. 2003; Heuertz et al. 2004; Lefort et al. 2000; Morand-Prieur et al. 2003; Morand-Prieur et al. 2003; Þvingila et al. 2005
Juglans	AFLP, isozymes, ISSR, microsatellites, RAPD, RFLP, SSR	Abuín et al. 2006; Aradhya et al. 2007; Dvorak et al. 2007; McGranahan 2007; Pollegioni et al. 2006, 2009; Potter et al. 2002; Ross-Davis et al. 2008a,b; Victory et al. 2006
Liquidambar	cpDNA	Morris et al. 2008
Liriodendron	ISSR, microsatellites, SSR	Liang et al. 2008; Xu et al. 2006; Yao et al. 2008
Platanus	ISSR, RAPD, RFLP	Besnard et al. 2002; Huang et al. 2009; Moore et al. 2006; Sun et al. 2009
Prunus	AFLP, cpDNA, microsatellites, QTL, RAPD, SNP, SSR	Clarke and Tobutt 2003; Dirlewanger et al. 2008; Dondini et al. 2007; Downey and Iezzoni 2000; Jordano and Godoy 2001; Olmstead et al. 2008; Pairon et al. 2006a, b; Pairon et al. 2008; Petitpierre et al. 2009; Van Huylenbroeck et al. 2004; Vaughan and Russell 2004
Quercus	AFLP, cpDNA, Isozymes, microsatellites, QTL, RAPD, RFLP, SSR	Aldrich et al. 2002, 2003, 2005; Austerlitz et al. 2007; Barreneche et al. 2004; Birchenko et al. 2009; Brendel et al. 2008; Burg et al. 1993; Casasoli et al. 2006; Cavender-Bares and Pahlich 2009; Cervera et al. 2000; Coart et al. 2002; Gailing et al. 2008; Hipp and Weber 2008; Hornero et al. 2001; Ishida and Kimura 2003; Lepais et al. 2009; López-Aljorna et al. 2007; Magni et al. 2005; Ohsawa et al. 2007a, b; Ohsawa and Ide 2008; Parelle et al. 2007; Petit et al. 2002; Pluess et al. 2009; Romero-Severson et al. 2003; Sisco et al. 2009; Valbuena-Carabaña et al. 2005; Wilhelm 2000
Robinia	AFLP, microsatellites	Huo et al. 2009; Mishima et al. 2009; Park and Han 2003; Yang et al. 2003
Tilia	AFLP, RAPD	Mylett et al. 2007; Van Huylenbroeck et al. 2004
Ulmus	AFLP, ISSR, RAPD	Goodall-Copestake et al. 2005; Pooler and Townsend 2005; Whiteley et al. 2003

AFLP amplified fragment length polymorphism, *ISSR* inter-simple sequence repeat, *QTL* quantitative trait loci, *RAPD* random amplified polymorphic DNA, *RFLP* restriction fragment length polymorphism, *SCAR* sequence characterized amplified region, *SSR* simple-sequence repeat, *SNP* single nucleotide polymorphism

foreign genes into many plant and tree species. For fine hardwoods, stable and cost-effective methods must still be developed for many species while a lack of long-term field trial data stall more progressive advances (Ahuja 2009). Without long-term data, commercialization of generated transgenics is unlikely to occur for many years (Table 1).

Agrobacterium-mediated transformation has been used extensively to study a number of aspects of hardwood tree species. Liu and Pijut (2010) describe a method for studying reproductive sterility in black cherry. Studies with silver birch looked at overexpression of 4-coumarate:coenzyme A ligase, a lignin biosynthesis gene involved in interactions between roots and soil microorganisms (Seppänen et al. 2007). Overexpression of sugar beet chitinase IV in silver birch decreased plant material palpability to insect foragers while increasing resistance to fungal disease (Vihervuori et al. 2008; Pasonen et al. 2009). American chestnut has been transformed to express the wheat germin-like oxalate oxidase gene in an effort to bolster its resistance to fungal pathogens (Welch et al. 2007). With hardwood genetic modification,

protocol optimization remains a challenge. In transgenic white birch, Zeng et al. (2010) examined the effects of repeated sub-culturing on gene stability in micropropagated plants, and noted a 22.5–65% decrease in transgene activity after the fifth subculture as a result of DNA methylation. Silencing could be reversed upon administration of 5-azacytidine, a DNA de-methylation agent (Zeng et al. 2010). Other studies have examined caveats within standard *Agrobacterium* transformation protocols in hopes of improving transformation efficiencies. Wang et al. (2009) looked at the role of kanamycin on hybrid walnut (*J. hindsii* x *J. nigra*) growth and noted that concentrations of 0–70 mg L^{-1} had relatively mild effects on transformation of nodal explants while growth and regeneration were compromised at >70 mg L^{-1} after 12 wk. In black cherry, kanamycin selection immediately after co-cultivation yielded zero regenerated shoots (Liu and Pijut 2010), while a 27-d late selection strategy allowed for transgenic development. Renying et al. (2007) showed that Chinese sweetgum was unaffected by the use of cefotaxime, used to inhibit *Agrobacterium* growth after infection in selection media, while Li et al. (2007) report acute sensitivities to all antibiotics used in the transformation process (i.e., cefotaxime, kanamycin, and timentin).

Use of wounding to enhance transformation efficiencies proved unnecessary with London plane tree (Li et al. 2007) and American chestnut (Rothrock et al. 2007), while Du and Pijut (2009) reported that wounding explants increased transformation efficiencies in green ash. In somatic embryo transformation, wounding was not necessary, as it may induce embryogenic tissue to generate callus rather than new somatic embryos (Rothrock et al. 2007). In American chestnut, wounding lowers transformation efficiency and encourages callus formation, a critical error, as regeneration from callus in American chestnut has not been described, and thus preserving the embryo-forming potential is imperative for whole plant regeneration (Rothrock et al. 2007). One of the first woody plants transformed to express foreign genes, walnut (*Juglans* spp.) regenerative successes generally result from embryogenesis rather than organogenesis, as the species is highly recalcitrant (Britton et al. 2007). After extensive optimization of the black locust transformation protocol, Shen et al. (2008) were able to generate up to 26% efficiency.

Additional conditions shown to affect transformation efficiency were inoculation and pre-culture time (Álvarez and Ordás 2007; Corredoira et al. 2007; Li et al. 2007; Shen et al. 2008; Andrade et al. 2009; Du and Pijut 2009; Liu and Pijut 2010), *Agrobacterium* OD_{600} (Álvarez and Ordás 2007; Li et al. 2007; Shen et al. 2008), and tissue type (Britton et al. 2007; Corredoira et al. 2007; Renying et al. 2007; Shen et al. 2008). In black locust, Shen et al. (2008) noted a variation in sensitivity to antibiotics with respect to tissue type, with stems being more sensitive than cotyledons. Use of somatic embryos at the globular stage rather than cotyledonary stage has been shown to increase transformation efficiencies in American chestnut (Corredoira et al. 2007; Rothrock et al. 2007).

Simple co-cultivation has been the primary method of generating transformants, however new variations of the method are emerging. Both Andrade et al. (2009) and Du and Pijut (2009) showed that addition of vacuum infiltration improved transformation efficiencies, with Du and Pijut (2009) utilizing a sonication step as well. Rothrock et al. (2007) used a new procedure termed "plate flooding" to ensure that only transgenic somatic embryos multiply while all others die. Comparing this to two other non-wounding and three wounding methods, plate flooding was as efficient as other more well-known methods. In plate flooding, somatic embryos were incubated with *Agrobacterium* while plated on multiplication medium, then the *Agrobacterium* was removed, and the embryos were co-cultivated in place (Rothrock et al. 2007). The use of embryogenic suspension in conjunction with vacuum infiltration and selection with Geneticin reported by Andrade et al. (2009) with American chestnut indicated a near 100% efficiency rate. By incubating size-fractionated proembryogenic masses in 2, 4-D for 2 d before *Agrobacterium* inoculation, antibiotic selection difficulties were resolved, and the use of Geneticin prevented development of escapes. Andrade et al. (2009) have generated a protocol that negates the need for reporter genes allowing only particular genes of interest to be incorporated into the American chestnut genome. Many "firsts" have been produced in genetic transformation of tree species in recent years. Renying et al. (2007) reported *Agrobacterium*-mediated transformation of Chinese sweetgum, Du and Pijut (2009) presented a transformation protocol for green ash, and Álvarez et al. (2009) reported on genetically engineering herbicide tolerance in cork oak with the introduction of the *bar* gene. New, more efficient plant transformation methods have been devised and implemented (Rothrock et al. 2007; Andrade et al. 2009). Many of these authors only experimented with one *Agrobacterium tumefaciens* strain, generally AGL1 or EHA105, and it is likely that greater optimization may be possible with another strain for a given tree species (Álvarez and Ordás 2007).

Non-timber Forest Products Derived from Trees

The term "non-timber forest product" (NTFP) usually describes: the fungi, herb, lichen, moss, shrub, tree, or vine; the parts of the plant (bark, branch, fruit, leaf, root,

sap and resin, tuber, twig, and wood); and other biological material harvested from the forest that are not timber based (Chamberlain et al. 1998, 2002). These commercial and non-commercial products may be utilized for chemical, cosmetic, culinary, floral and decorative, dietary, drug, manufacturing, medicinal, and wood-based purposes (Chamberlain et al. 1998, 2002; Omar et al. 2000; Ciesla 2002; Jones et al. 2002; Turley et al. 2006). There are numerous books, websites, databases, journal articles, and other types of publications that discuss NTFPs; but in this review, we will try to briefly summarize some of the non-wood products obtained from important hardwood tree species. Biotechnological tools can be utilized to improve and preserve some of these valuable resources, and research in this area needs further development.

Bark. The outer bark of cork oak is hand harvested (stripped) for commercial products. A cork tree can regenerate its outer layer 12 or 13 times during its lifetime, and cork is usually harvested in 10-yr intervals (see for example, Cork Institute of America 2010). Cork planks and granulated cork waste are used in the manufacture of diverse products, such as building materials, bulletin boards, gaskets, insulation, tapered stoppers, wine or champagne closures, duck decoys, flooring, and fishing floats and rod handles. Tannins derived from the bark of chestnut, oak and some species of alder are used for tanning animal hides (skins) in the production of leather for various products (see for example, Pergamena 2010). The bark from several temperate species are used as traditional natural dyes, producing various colors on wool, cotton, and silk fabrics, when used alone or combined with a mordant (Ciesla 2002; Jones et al. 2002). Salicin, a glycoside which acts as a precursor compound for the synthesis of acetyl salicylic acid, the active ingredient in the bark of willow (*Salix* spp.) was traditionally used to relieve pain (Ciesla 2002). The bark of paper birch is still used today for hand-made baskets, in the floral industry, for novelty products, and for the extraction and conversion of betulin to betulinic acid for cosmetic, pharmaceutical and manufacturing purposes (see for example, Soft Protector Ltd. 2010). Sweet birch essential oil distilled from the bark of sweet birch is sold as specialty oil for aromatherapy. The aromatic bark (and roots) of sassafras are used to make a medicinal tea, but safrole-containing food additives were banned by the US Food and Drug Administration in 1960 after safrole was found to be hepatocarcinogenic (liver cancer).

Bonsai. Bonsai, the ancient oriental horticultural art form of pruning, shaping, and training a plant into a miniature of a fully grown mature tree, can be achieved with many different hardwood trees (*Acer*, *Alnus*, *Betula*, *Carpinus*, *Carya*, *Castanea*, *Celtis*, *Fagus*, *Fraxinus*, *Juglans*, *Liquidambar*, *Liriodendron*, *Platanus*, *Prunus*, *Quercus*, *Salix*, *Sorbus*, *Tilia*, and *Ulmus*). Many species are chosen for bonsai because of the colorful foliage, seasonal flowers, or hardiness. Species used for bonsai need to be able to produce new buds on old wood, thereby allowing the continual pruning and shaping to occur. Other preferred qualities include small-leaved varieties, short internodes, and the ability to withstand root disturbance. The appearance of old age of a bonsai tree is aesthetically pleasing and highly prized, and specimens may live for many years.

Flowers and foliage. The flowers of several tree species (e.g., *L. tulipifera* L. [tuliptree], *Nyssa ogeche* Bartram ex Marsh. [Ogeechee or white tupelo], *Oxydendrum arboretum* [L.] DC. [sourwood], black locust and *Tilia americana* L. [American basswood]) are an excellent source of nectar and sweet deposits that are gathered, modified, and stored in the honeycomb by honey bees (Ciesla 2002; Jones et al. 2002; National Honey Board 2010). The dried and ground leaves of sassafras (*Sassafras albidum* [Nutt.] Nees), called "File", is used in traditional Creole or Cajun cooking for flavoring and thickening. The foliage of some hardwood species is used in various floral decorations and occasionally used as fodder for cattle (Ciesla 2002; Jones et al. 2002).

Fruits. The fruit from European mountain ash (*Sorbus aucuparia* L.) is used in the production of jams, jellies, cider, as sun-dried berries and as a flavoring for wine (Ciesla 2002). Fruits of the common persimmon (*Diospyros virginiana* L.) are used in cookies, cakes, puddings, custards, and sherbet (USDA-NRCS 2010).

Nuts. Mast production from hardwood tree species provides an important food source for many types of wildlife (amphibians, birds, mammals, and reptiles) and as a source of feed in the specialty production of Spanish hogs (cork oak acorns and Iberian hogs). Hard mast refers to seed with a hard shell (black walnut, hickory, oak) and soft mast refers to seed with a fleshy covering (black cherry, sassafras). Many of the nuts are relatively high in carbohydrates, fat, protein, vitamins, and minerals (Duke 2010), and they have a long history of uses (Leopold et al. 1998; Ciesla 2002; Jones et al. 2002; USDA-NRCS 2010). Some nut crops for human consumption include almonds, chestnuts, hazelnuts (filberts), pecans, pistachios and walnuts (black and English), and are commercially grown in orchards. Crushed acorns from oak and the hulls of nuts from walnuts are a source of dye and produce a range of colors (Ciesla 2002; Jones et al. 2002). Ground nutshells of black walnut are used as a soft grit abrasive for cleaning and polishing metal, fiberglass, wood, plastic, and stone

and in cosmetics; in the oil drilling industry to maintain seals and filtration; and as fillers and extenders in the dynamite industry (see for example, Hammons Products Co. 2010). Gourmet cooking oils (walnut and pecan) are also commercially produced.

Sap and resin. Maple sap, mainly from sugar maple trees (*Acer saccharum* Marsh.) and other maple species, is harvested and much of the water evaporated for the production of maple syrup. One (20 g) serving of 100% sugar maple syrup is rich in energy (52 kcal), carbohydrate (13.4 g), total sugar (11.9 g), calcium (13 mg), magnesium (3 mg), potassium (41 mg), zinc (0.83 mg), and iron (0.24 mg; USDA 2010). Sap collected from paper birch in Alaska is used in the specialty production of birch syrup; even though the sugar (fructose) content of birch sap is about one-third that of maple sap (sucrose; see for example, Kahiltna Birchworks 2010). Sap is also used in the production of maple creams and candies, and in birch beer (*Betula lenta* L.; sweet birch; Ciesla 2002). Styrax gum (resin) from *L. styraciflua* L. (sweetgum) is steam distilled and the fragrant oil used in the drug and cosmetic industries (Ciesla 2002).

Case Studies

Merkle et al. (2007) provided a thorough review of both conventional genetic improvement and biotechnological approaches to restoring threatened forest tree species, including a discussion of public perception and regulatory hurdles. Thus, in our case studies below, we will limit our focus to biotechnological efforts to preserve three forest tree species that are native to the USA: American chestnut, American elm, and North American ash.

American chestnut. American chestnut (*C. dentata*) was once a dominant hardwood forest species in the eastern USA before it was mostly eliminated by the introduced chestnut blight fungal pathogen (*Cryphonectria parasitica* [Murrill] Barr) in the early 1900s (Burnham 1988; Griffin 1986). Before the twentieth century, this species provided much of the wood construction materials for housing and farmstead uses, wood for other household uses such as bowls and eating utensils, as well as sustenance for humans and farm animals provided by the nuts. Wildlife depended upon the trees' nutritional annual nut crop for nourishment and building body fat before winter. After devastation from the blight, American chestnut was maintained in the forest understory and as individual specimens in collections, because of the trees ability to re-sprout from the root collar after dieback (Beattie and Diller 1954), and in some cases, trees escaped infection from the disease. Either through backcross breeding to transfer blight resistance from Chinese chestnut (*C. mollissima*; Bernatzky and Mulcahy 1992), now in widespread practice through The American Chestnut Foundation and its partners, or through bioengineering for blight resistance (Powell et al. 1995), the diversity of this species can potentially be maintained. Even more importantly, Jacobs and Severeid (2004) reported that American chestnut had the potential to provide great economic value because of fast growth, good timber quality, and early mast production in field trials of unimproved germ plasm grown in an area where the pathogen had yet to infect the trees; showing potential performance that could be expected in future restoration efforts.

For applications in bioengineering, Carraway and Merkle (1997) and Xing et al. (1999) built upon earlier work by Merkle et al. (1991) and reported on an *in vitro* protocol for production of American chestnut somatic embryos (SE). They found 2,4-D to be necessary for repetitive SE production, and BA at higher concentrations than that found to be optimal reduced induction of SE. Acclimatization of SE plants was problematic requiring additional improvement of embryo maturation and conversion in later studies. In the same lab, Robichaud et al. (2004) were able to improve germination rate and taproot length with the addition of asparagine to the culture medium. Johnson et al. (2008) made further improvements to SE plant production by increasing cold treatments >12 wk, and treating mature SE with red light. With all of these cultural improvements, Andrade and Merkle (2005) reported that several transgenic lines could be routinely regenerated by SE in liquid culture in large numbers. Andrade et al. (2009) then reported that they used this liquid suspension system to produce more than 100 transgenic seedlings using *Agrobacterium*-mediated transformation containing marker genes, and these trees flowered 3-yr *ex vitro*. Using a semi-solid culture medium, Rothrock et al. (2007) used plate flooding with *Agrobacterium* to transform American chestnut SE, and whole plants were regenerated from one line.

Others have focused on *in vitro* shoot propagation as a system for deployment of genetically superior and bioengineered trees. Early investigators (Keys and Cech 1982; Serres et al. 1990) had success with regeneration of microplants from juvenile plant material, and Read et al. (1985) and Read and Szendrak (1995) were successful starting with stem segments from mature trees. Maynard et al. (1993) and Xing et al. (1997) made improvements to these earlier protocols, with the latter group making slight improvements to help eliminate shoot tip necrosis, a perennial problem during adventitious rooting. Yang et al. (2009) performed an extensive study using auxin and cytokinin combinations to enhance both adventitious shoot

and root initiation, and found that N-(2-chloro-4-pyridyl)-N′-phenylura and TDZ were effective at low concentrations for enhanced shoot regeneration.

Pollen transformation, as a means to transfer resistance to progeny, has been explored (Fernando et al. 2006). In this study, the authors tested parameters necessary for microparticle bombardment, including target distance, target pressure and receptive pollen development, which led to transient expression of fluorescent marker genes. Carraway et al. (1994) reported the first stable transformation via *Agrobacterium*, but without regeneration of plants. Powell et al. (2005) and Polin et al. (2006a) bioengineered trees containing an oxalate oxidase gene from wheat. These trees are currently in field tests to determine the gene's ability to confer resistance to the chestnut blight. Welch et al. (2007) described potential disease resistance mechanisms mediated by the transgene oxalate oxidize through studies with wild-type and transgenic callus tissue. It was concluded that oxalate oxidase helps prevent changes in cell wall composition following infection by *C. parasitica*.

One approach that should be considered is to combine the efforts of those working in transgenic technologies with the use of genetically improved and genetically diverse American chestnut lines that are under development by numerous volunteer groups in association with The American Chestnut Foundation. Working alone, there is no guarantee that either effort will be successful in producing blight-resistant plant material, but together the odds of success might be dramatically increased. Transgenic technologies are often limited to improvement of a few genotypes, and classical backcross breeding approaches are counting on yet to be proven resistance mechanisms that can be maintained long term.

American elm. American elm (*U. americana*) was once a dominant feature of the eastern urban forests, particularly along streets and boulevards in the early twentieth century. An introduced fungal pathogen, *Ophiostoma ulmi* (Buisman) Nannf., vectored by the native elm bark beetle [*Hylurgopinus rufipes* (Eichhoff)] and the European elm bark beetle (*Scolytus multistriatus* Marsh.), caused the loss of the majority of these trees as a result of Dutch elm disease (DED) that resulted from infection (Hubbes 1999). To complicate the matter, a highly aggressive strain, *Ophiostomo novo-ulmi*, has been described (Brasier 1994).

American elm had been prized as a street and park tree because of its broad, vase-shaped habit, longevity in the landscape and forest, and tolerance of urban conditions such as drought and pollution (Dirr 1998). Since its demise, no comparable replacements for this species have been found, but *Fraxinus* spp. and hybrid elms have become suitable replacements until now. With the introduction of the emerald ash borer into the USA, this insect pest now threatens all *Fraxinus* spp.

Gautheret (1940) first reported on adventitious shoot regeneration in the early days of plant tissue culture. Durzan and Lopushanski (1975) were successful in regenerating both roots and shoots from American elm callus. It was much later when McCown and McCown (1987) reported on commercial production of elms by axillary shoot proliferation. Chanon et al. (1997) were successful with regeneration of superior American elms from both seedlings and mature trees using hypocotyls and nodal stem segments. During that intervening time, shoot regeneration from leaf explants was reported (Bolyard et al. 1991b; Bolyard 1994; George and Tripepi 1994).

Eshita et al. (2000) reported on the development of a cell suspension culture system for American elm. Rather than for use in transformation and plant regeneration, this group was interested in using the system for elicitor treatment experiments for determination of disease responsive genes. Aoun et al. (2009) and Nasmith et al. (2008) later implicated a role for phenyalanine amnonia lyase, chitinase, and polygalacturonase-inhibiting protein in possible resistance to *Ophiostoma* spp.

Initial attempts at both microprojectile and *Agrobacterium*-mediated transformation were reported for an American elm cultivar (Bolyard et al. 1991a). Although not with American elm, Corredoira et al. (2002) described production of SE lines from both *U. minor* and *U. glabra*, establishing a regeneration system that could be responsive to transformation. In the intervening decade after the Bolyard report, despite several years of similar research activity in Europe with *U. procera* (Fenning et al. 1996: Gartland et al. 2000, 2001, 2005), Newhouse et al. (2006, 2007) reported successful transformation of American elm leaf pieces with ESF39A, an antimicrobial peptide driven by a vascular promoter from American chestnut. To date, the transgene appears to help exclude the pathogen from vascular tissue and greatly reduces vascular staining. To further address possible regulatory concerns, the construct was designed to enhance mammalian digestion of the transgene. This work seems very promising for enhancing DED resistance, especially since work in Western Europe has ceased because of government intervention.

In lieu of DED-resistant American elms produced by biotechnological means, the USDA selected two cultivars with various levels of disease tolerance (Kuser and Polanin 2001). They include "New Harmony" and "Valley Forge," and those cultivars seem to withstand the disease better than "Princeton," which was selected by the Princeton Nurseries in 1922. Those cultivars, along with four other hybrid elms, were field tested in more than four sites throughout the native range of elm.

Although funding for transgenic research with American elm is very limited, both the need for restoration with DED-resistant germ plasm and the public interest to see this work proceed is great. At one time, American elm maintained an important urban and rural forest niche, providing both aesthetic and ecological values. The void that has been left from the absence of this species in those ecosystems has not been filled and furthermore, biodiversity in the USA could effectively suffer another loss without intervention from conventional or biotechnological means.

North American ash. North American ash species (*Fraxinus* spp.), including white ash (*F. americana*) and green or red ash (*F. pennsylvanica*), are important forest, park and urban trees, but they now are threatened by the emerald ash borer (EAB; Haack et al. 2002). In addition, the minor ash species, blue ash (*Fraxinus quadrangulata* Michx.), pumpkin ash (*Fraxinus profunda* (Bush) Bush), and black ash (*F. nigra*) are threatened because no known genetic resistance to EAB exists in any of the native North American species (Du and Pijut 2009). The emerald ash borer, a recent exotic insect introduction from Asia, is spreading rapidly throughout the East as a result of the movement of infested firewood; and near the source of the initial infestation in Michigan, more than 25 million ash trees have been killed (Cappaert et al. 2005). The potential cost of the loss of all urban ash in the USA could reach $2.4 trillion, and for ash in the natural forests, $282 billion (Sydnor et al. 2007). Wood from these species has numerous uses, including furniture, tool handles, pallets, crates, and in specialized cases for baseball bats (*F. americana*) and basketry (*F. nigra*). Ironically, as an urban street tree, some of the major ash species have been widely used as a replacement for American elm, a tree that once dominated the urban landscape. Now, most likely, a replacement for ash will also need to be found.

Van Sambeek and Preece (2007) provided a thorough review on *in vitro* propagation of many North American and European ash species, so this case study will focus on more recent uses of biotechnology for use in ash conservation and enhancing biotic resistance. In summary, for the major ash species, axillary shoot production from zygotic embryos or cut seeds was stimulated when thidiazuron was supplemented in the culture medium. Rooting of axillary shoots occurred in the presence of NAA and IBA in lower salt medium, but these synthetic auxins were not always necessary. Both greenhouse and field trials performed by numerous authors have shown negligible phenotypic variation arising from *in vitro* culture.

For green ash, Du and Pijut (2008) reported successful adventitious shoot regeneration of plants from cotyledons and hypocotyls using isolated zygotic embryos. Rooted *in vitro* plants were overwintered in cold storage, and they suggested that their protocol could be used as a platform to incorporate transgenes that infer resistance to the EAB. One year later, the same authors (Du and Pijut 2009) reported transformation of this ash species with two marker genes [β-glucuronidase (GUS) and an enhanced green fluorescent protein], and an antibiotic resistance gene [neomycin phosphotransferase]. In addition, they mentioned that experiments were under way to introduce the gene for a Cry8Da protein from *Bacillus thuringiensis* into green ash hypocotyls. Earlier, Kim et al. (1997, 1998) had reported on axillary shoot proliferation and rooting protocols for three clones of green ash, and that system has potential for mass propagation of elite transgenic genotypes once such trees have been produced.

For white ash, Bates et al. (1992) reported that the use of thidiazuron stimulated both shoot organogenesis and somatic embryogenesis of seed explants, and this was a significant improvement over earlier work reported by the same group (Navarrete et al. 1989). Adventitious shoots and epicotyls from germinated somatic embryos were rooted and acclimatized under greenhouse conditions. Pijut (personal communication) reports adventitious shoot regeneration from hypocotyls and cotyledons, plus the production of acclimatized plants to the greenhouse. It is likely that this protocol will be beneficial for transgenic studies.

For black ash, Pijut (personal communication) has recently reported successful regeneration of adventitious shoots from hypocotyls and production of acclimatized plants to the greenhouse, and production of GUS-positive transgenic callus with shoots that grew on normally lethal doses of kanamycin. Thus, future transgenic work with black ash has potential to make inroads with enhancing host resistance to EAB.

Work by Brearley et al. (1995); Schoenweiss et al. (2005), and Ozudogru et al. (2010), although with common ash in Europe (*F. excelsior* L.), suggests that cryopreservation of zygotic embryos, embryogenic callus and shoot tips could be an effective tool for conservation of North American ash species. Recently, Volk et al. (2009) were successful with germplasm conservation of numerous North American species by cryopreserving dormant buds. These authors grafted the thawed buds on rootstocks, and growth resumed with high success rates for all the species tested.

Unless biocontrol measures, such as fungal entomopathogens (Castrillo et al. 2010) or parasitoids (Ulyshen et al. 2010), can be found to be effective against EAB, or some host resistance becomes evident, transgenic approaches may be the only pathway to obtain EAB-resistant ash germplasm for future restoration efforts. In the meantime, seed collections, long-term seed storage, and cryopreservation of shoot tips and buds will help to retain the diversity

within each species until such time that restoration can proceed.

Conclusions

The use of biotechnological approaches for the conservation, enhancement, genetic improvement, and restoration of temperate hardwood forest tree biodiversity has advanced considerably in the last 10 yr. Trees are invaluable resources and provide economic, ecological, aesthetic, and spiritual value that must be conserved in a sustainable fashion for future generations. Forest tree biotechnology offers the potential to conserve germplasm of threatened and endangered tree species; develop trees with disease and pest resistance; improve the growth, form and wood properties of trees; aid in single species restoration and land reclamation; and aid in the development of new commercial products that have yet to be discovered from trees. For genetically modified trees to be deployed commercially and improve rather than threaten biodiversity, future studies in transgenic technology must focus on transgene expression and stability for both qualitative and quantitative traits, plus the prevention of transgene escape to native populations. We must also consider conserving the habitats and ecological processes within native temperate hardwood forests. The loss or destruction of habitat, habitat fragmentation and degradation, and climate change all have an impact on a tree species survival, and in turn affect the populations, species, and communities around them. The development of appropriate biotechnological conservation strategies for a number of hardwood tree species still needs further research and development. Conservation through biotechnology will have a global economic and ecological impact on sustaining forest tree biodiversity.

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