Somatic Embryogenesis and Cryostorage for Conservation and Restoration of Threatened Forest Trees[1]

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Threats to North American forest trees from exotic pests and pathogens or habitat loss, make it imperative that every available tool be employed for conservation and restoration of these at risk species. One such tool, *in vitro* propagation, could greatly enhance conservation of forest tree genetic material and selection and breeding of resistant or tolerant genotypes for restoration. *In vitro* propagation approaches include standard micropropagation (axillary shoot multiplication), organogenesis (adventitious shoot production) and somatic embryogenesis (SE), a process by which structures (somatic embryos) resembling seed embryos are produced asexually. The SE systems, in particular, are well-suited for conservation and restoration purposes, due to the high multiplication rates and the amenability of embryogenic cultures to cryostorage. Examples of threatened forest species for which we have developed SE systems that are already being applied for conservation and restoration efforts include American chestnut (*Castanea dentata*), eastern and Carolina hemlocks (*Tsuga canadensis* and *T. caroliniana*), Atlantic white cedar (AWC, *Chamaecyparis thyoides*), and green and white ash (*Fraxinus pennsylvanica* and *F. americana*). We have also developed an adventitious shoot-based propagation system for Franklinia (*Franklinia alatamaha*), which has been extinct in nature for over 200 years.

American chestnut, once one of the most important trees in eastern North America, was devastated by chestnut blight, caused by *Cryphonectria parasitica*, which was accidentally introduced from Asia in the late 1800s. Breeders in the American Chestnut Cooperators Foundation (ACCF) have been crossing large surviving American (LSA) chestnut trees to produce progeny with enhanced resistance, while The American Chestnut Foundation's (TACF) breeding program has focused on a hybrid backcross breeding program to introgress genes from the blight resistant Chinese chestnut (*C. mollissima*) into the American chestnut background. Over the past 25 years, we have developed an embryogenic culture system for American chestnut using immature seeds as explants (fig. 1A). Employing a suspension culture-based system, the cultures can be scaled-up to produce hundreds of clonal somatic seedlings from a given culture line (Andrade and Merkle 2005). The cultures are also highly amenable to cryostorage and recovery (Holliday and Merkle 2000). In collaboration with ACCF, we have used SE to propagate germplasm derived from crosses between LSAs so that clones can be tested for blight resistance. The first somatic seedlings from these cultures were planted on National Forest land in Virginia starting in 2012 and are growing well. We have also collaborated with TACF to implement clonal testing of accessions from their breeding program for resistance to chestnut blight. Control-pollinated BC3F3 seeds from selected BC3F2 seed orchard parents were used to initiate embryogenic cultures from which chestnut somatic seedlings have been produced for field testing (Holtz et al. 2017, Merkle et al. 2013). Copies of the BC3F3 cultures will be held in cryostorage until the clones with the best field performance are identified. These clones can then be recovered from cryostorage and scaled-up for production of planting stock. For restoration purposes, multiple, chestnut clones of different parentage will probably need to be developed to ensure genetic diversity and adaptation to regional conditions. Elite clones showing the fastest growth rates and/or superior wood quality, in addition to disease resistance, may someday be

[1] A version of this paper was presented at the Gene Conservation of Tree Species – Banking on the Future Workshop, May 16-19, 2016, Chicago, IL.

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deployed by landowners for timber production. We have also applied our chestnut SE protocol to produce the first embryogenic cultures of Ozark chinquapin (*Castanea pumila* var. *ozarkensis*), which like the American chestnut, has been severely affected by chestnut blight (Merkle et al. 2017).

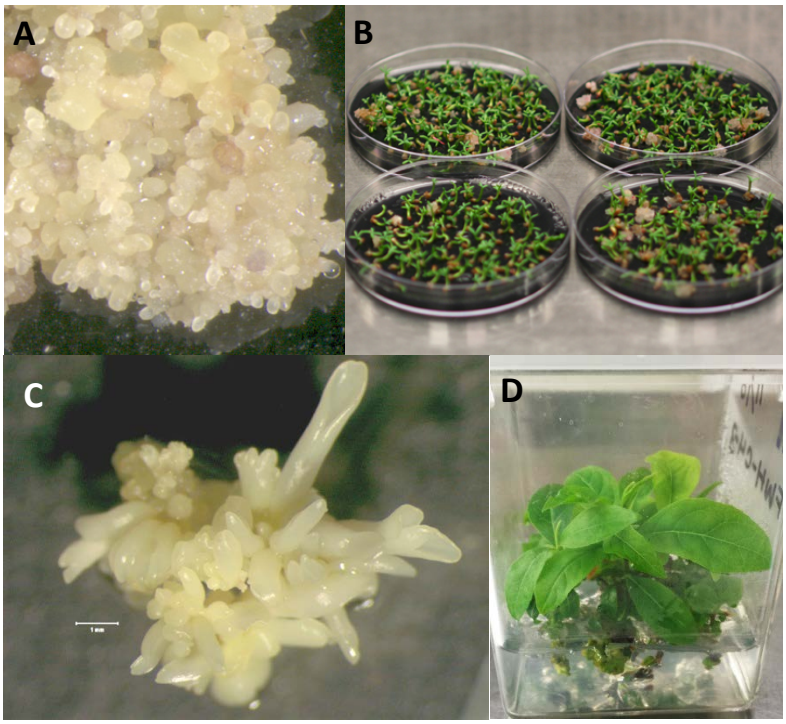

Figure 1—*In vitro* propagation of threatened and rare forest trees. A. American chestnut somatic embryos. B. Germinating Carolina hemlock somatic embryos. C. White ash somatic embryos. D. Franklinia shoot proliferation.

Both eastern hemlock and Carolina hemlock have suffered huge losses from hemlock woolly adelgid (HWA, *Adelges tsugae*) attacks, and genetic diversity is being lost in these species. While seeds of many forest tree species are amenable to long-term storage, this has not been demonstrated with seeds of hemlocks, different seed lots of which showed highly variable retention of viability when refrigerated for 2 to 4 years (Olson et al. 1959). Primarily as a means of conserving hemlock germplasm, we developed embryogenic and cryostorage systems for these two species by applying standard protocols that were successfully applied to other Pinaceae species (Merkle et al. 2014). More recently, we worked with breeders at North Carolina State University and the Forest Restoration Alliance (FRA) to apply SE to clonally propagate hybrids between Carolina hemlock and HWA-resistant Asian hemlock species and putatively HWA-resistant eastern hemlocks, with the goal of producing HWA-resistant hybrid hemlocks. Starting with immature seeds derived from hybrid crosses, we have produced multiple embryogenic cultures of hybrids between Carolina hemlock and Chinese hemlock and between Carolina hemlock and southern Japanese hemlock (*T. sieboldii*). Somatic seedlings have been regenerated from the cultures (fig. 1B) and transferred to FRA collaborators to grow up for eventual resistance screening. Some putatively HWA-resistant eastern hemlock individuals have been identified in the past few years. Working with collaborators from the New Jersey Department of Agriculture and the University of Rhode Island, we recently initiated cultures from seeds collected from one such tree growing in the “bulletproof” stand in New Jersey, so-called because the hemlocks in the stand have survived HWA infestation while surrounding hemlocks have all succumbed. The first somatic seedlings from these cultures should be produced soon.

While AWC populations in the eastern United States have not been attacked by exotic pests or pathogens, they have suffered dramatic declines due to over-harvesting, fire suppression, hydrologic alteration, and conversion of coastal bogs to agriculture and development. To aid in restoration efforts, we

have collaborated with Camcore personnel to develop a SE-based propagation system. Recently, using seeds collected by a Camcore collaborator in North Carolina, as well as trees planted on the University of Georgia, Athens campus, we produced the first AWC embryogenic cultures and showed that these cultures can be cryostored and recovered (Ahn et al. 2016). To date, a small number of AWC somatic seedlings have been produced, acclimatized and grown in the greenhouse, but as of yet, there are no plans to expand production of trees for field testing.

The valuable landscape and wood products trees white ash and green ash are under threat of extirpation from their native ranges by the emerald ash borer (EAB, *Agrilus planipennis*), an exotic wood-boring beetle that has already destroyed millions of ash trees in 15 states and Canada. Similar to the case with eastern hemlock, putative EAB-resistant ash individuals have been identified in native populations. Since these trees are found in areas where over 90 percent of the ash trees have been killed by EAB, they are called "lingering ash" trees. Applying a highly prolific SE system that we originally developed for green ash (Li et al. 2014), we are collaborating with Ohio State University scientists to initiate embryogenic cultures (fig. 1C) from seeds collected from multiple "lingering" white ash trees in Michigan. The first somatic seedlings from some of these "lingering ash" cultures have been produced and have grown rapidly in the greenhouse and shade house. We hope these will reach sufficient diameter in the next few years to be screened for EAB resistance, and may become the basis for new EAB-resistant varieties to be planted by landowners.

Franklinia, a member of the tea family discovered growing in a single population in Georgia by John and William Bartram in the 1700s, has not been seen in nature since 1803. Its reintroduction to the wild, or even as a landscape tree in the southeastern United States has been hindered by its extreme susceptibility to Phytophthora root rot (PRR), caused by *Phytophthora cinnamomi*, which is endemic throughout the southeast. As a first step to developing Franklinias that can survive PRR infection, either using mutagenesis or transgenics, we have developed an adventitious shoot-based regeneration system for the tree. Adventitious buds form on cultured immature zygotic embryos and rapidly elongate into shoots (fig. 1D) that are rooted in vitro and hardened off to greenhouse conditions.

All of the SE and other in vitro propagation systems we have developed for threatened or rare forest trees have benefitted greatly from collaboration with scientists in different organizations. Their continued development and eventual application similarly will rely on such collaborations. In fact, we contributed this paper to the Gene Conservation of Forest Trees meeting primarily in hopes of forging new collaborations with scientists working with threatened forest tree species. These systems are potentially very powerful conservation and restoration tools, but they are only of use when we have selectors, breeders, pathologists, entomologists, silviculturists and other scientists who will collaborate with us to test and apply the products of our work to addressing forest health challenges.

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