THE INFLUENCE OF CHINESE CHESTNUT GENOME PROPORTION ON THE SUCCESS OF SOMATIC EMBRYOGENESIS IN CHESTNUT

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The American chestnut (*Castanea dentata*) was one of the most economically important angiosperm forest tree species in the eastern United States up until the early 1900s (Carraway and Merkle 1997). The range of the species extended from northern Georgia and Alabama through southern Maine and parts of Canada (Burnham 1988). Comprising up to 25 percent of the trees in the Appalachian forest and with a natural life expectancy of 500-1000 years, the American chestnut could reach up to 150 feet tall and 10 feet in diameter. The tree was fast-growing and its wood was highly rot-resistant and did not warp or shrink, thus making it a major timber tree for construction lumber and furniture (Andrade and Merkle 2005; Vieitez 1995; Anagnostakis 1987). The nuts that the tree produced were an essential cash crop. A single tree could produce over 100 kilograms of nuts annually at maturity (Vieitez 1995).

Around 1904, the chestnut blight fungus (*Cryphonectria parasitica*) was introduced to the United States on Asian chestnut nursery stock. *C. parasitica* is necrotrophic, producing a sunken canker that eventually girdles the tree. The fungus spread approximately 200 miles every 10 years, and it took only 45 years for 9 million acres of American chestnut trees to be either infected or dead (Anagnostakis 1987; Burnham 1988; Johnson et al. 2008; Vieitez 1995). Today, the tree can still be found in the understory due to its stump sprouting ability, but rarely does it survive beyond a few years. Natural reestablishment of the American chestnut is highly unlikely, as the blight remains an obstacle.

As a part of the considerable effort to re-introduce the American chestnut to its native range, a significant amount of research has been put forth to produce and propagate American chestnut trees with blight resistance. One such approach is hybridization with Asian chestnut trees, which carry resistance to the fungus. Burnham (1988) suggested that crossing an American chestnut with a Chinese chestnut and then backcrossing multiple times to American chestnut would result in an individual with both American chestnut characteristics and the genes from Chinese chestnut responsible for blight resistance. The American Chestnut Foundation (TACF) was established to accomplish the plan that Burnham proposed. Since a system has already been developed to clonally propagate pure American chestnut trees via somatic embryogenesis (SE; Carraway and Merkle, 1997; Andrade and Merkle 2005), we believe it also has great potential to be used to multiply blight-resistant trees produced by TACF's hybrid backcross breeding program.

Since it is well-known that genotype exerts a strong influence in embryogenesis induction, it is possible that Chinese chestnut (CC) genes in the hybrid backcross material could affect the success of SE using the protocol established for American chestnut (AC). To date, there have been no published reports of somatic embryogenesis in either CC or hybrid backcross material. Over three years of culture initiations, we tested the effects of CC genome proportion on the success of SE induction using our standard protocol for culturing AC and, subsequently,

protocols based on a published protocol for SE in European chestnut (Vieitez 1995). A total of 145 different chestnut genotypes were used to assess the impact of hybrid genotype on the success of embryogenesis induction. As shown in Table 1, along with American chestnut and Chinese chestnut, tested genotypes included the following CC x AC hybrid and backcross types: F1 (½ Chinese and ½ American), B1 (¾ American and ¼), B2 (7/8 American and 1/8 Chinese), B3F3 (15/16 American and 1/16 Chinese). In 2010, all of the species and hybrids were cultured using the standard protocol. Briefly, burs were dissected to remove the nuts and the immature seeds were cultured in on semi-solid induction-maintenance medium (IMM; Andrade and Merkle 2005), which is a modified woody plant medium (WPM; Lloyd and McCown 1980) with 4.0 mg l^{-1} 2,4-D, 0.5 g l^{-1} L-glutamine, and 3% sucrose. All cultures were transferred to fresh IMM of the same type after one month, and at the end of the second month, they were transferred to secondary medium, which was the same as IMM, but with only 2.0 mg l^{-1} 2,4-D. Cultures were scored for signs of proliferation of embryogenic material after 10 weeks and the percentage of seeds that produced embryogenic material was calculated for each source. None of the CC or F1 cultures produced embryogenic tissue; however, the B3F3 and American chestnut explants had inductions of 0.99% and 1.60% respectively.

Table 1. Hybrid types cultured each year of the experiment

2010	2011	2012
American chestnut	American chestnut	American chestnut*
B3F3	B3F3	B3F3*
F1	Chinese chestnut*	B2*
Chinese chestnut		B1*
		F1*
		Chinese chestnut*

* Species and hybrids cultured using both the standard protocol and experimental treatment

In 2011, 3 new treatments were tested for the CC initiation, along with the standard protocol. Research conducted on the phylogeny of *Castanea* shows that the genus originated in Asia, migrated west to Europe, and then to North America (Lang et al. 2007). Based on these data, we theorized that CC is more closely related to European chestnut (*Castanea sativa*) than AC and that induction protocols based on the one reported for European chestnut by Vieitez (1995) may be successful for CC embryogenesis induction. Nuts of each genotype were randomly divided into four groups and seeds were cultured using one of four treatments (Table 2). CC cultures initiated with Treatment 4 were the only cultures to successfully produce embryogenic tissue with a percent induction of 1.19. In 2012, only two treatments were used for the initiation of all species and hybrid types: the successful experimental treatment (Treatment 4) from 2011 and the standard protocol. With the standard protocol, induction percentages were as follows: AC, 2.35; B3F3, 0.47; B2, 0.80; B1, 0; F1, 0.36; CC, 0. Cultures initiated with the introduced treatment had the following induction percentages: AC, 0; B3F3, 0; B2, 0.77; B1, 0; F1, 0; CC, 2.04.

Analysis of variance results for all three years of initiation data combined, for the standard protocol only, indicated there were significant differences among the species and hybrids ($P = 0.0037$). Tukey's test revealed that AC and B3F3 were not significantly different from each other, but both were significantly different from CC. When all hybrid types as well as the AC

and CC cultures were compared, using both the standard protocol and Experimental Treatment 4, analysis of variance results showed that species and hybrids were significantly different ($P < 0.001$), and the interaction between species/hybrid and media treatment was also significant ($P = 0.0221$). The results of these experiments indicate that the proportion of Chinese chestnut genome does have an effect on the induction of SE using the standard protocol for American chestnut. The significant interaction between species/hybrid and treatment is due to the fact that the standard SE protocol works for pure American chestnut, and B3F3 and B2 hybrids, but not Chinese chestnut, B1, or F1 hybrids, while the European chestnut SE protocol was successful for Chinese chestnut, but not for other Chinese x American hybrids.

Table 2. Experimental treatments used for Chinese chestnut culture initiations in 2011

Primary medium	Secondary medium
*1. WPM with 4 mg/l 2,4-D	WPM with 2 mg/L 2,4-D
2. WPM with 4 mg/l 2,4-D	WPM with 0.1 mg/l BAP and 0.05 mg/l NAA
3. WPM with 1 mg/l 2,4-D and 1mg/l BAP	WPM with 1 mg/l 2,4-D and 1 mg/l BAP
*4. WPM with 1 mg/l 2,4-D and 1mg/l BAP	WPM with 0.1 mg/l BAP and 0.05 mg/l NAA

* Treatments also used in the 2012 culture initiations

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