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# Is Nut Cold Tolerance a Limitation to the Restoration of American Chestnut in the Northeastern United States?


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American chestnut (*Castanea dentata*) was once a dominant hardwood species in the eastern United States, growing from Maine to Georgia and west to the Ohio Valley (Harlow et al. 1979). Arguably, American chestnut may have been the most important hardwood species in North America, renowned for its quick growth, massive size, and great utility (Harlow et al. 1979). Unfortunately, within 50 years of the introduction of chestnut blight (*Cryphonectria parasitica*)—a fungal disease native to Asia—American chestnut was functionally removed as an overstory tree from eastern forests (Griffin 2000).

Owing to the economic and ecological value of American chestnut, several methods of species restoration have been attempted, including biological control of chestnut blight through pathogen hypovirulence, and intra- and interspecific breeding (Griffin 2000). Of the methods attempted thus far, backcross breeding has shown the most promise for near-term, comprehensive species restoration (Griffin 2000). Backcross breeding involves an initial cross of American and Asian chestnut (usually Chinese [*Castanea mollissima*]), followed by backcrossing hybrid offspring with additional American chestnut parents, which increases the proportion and diversity of American chestnut genes in the breeding pool. The goal is to breed a tree that is extremely similar in genotype and phenotype to American chestnut while capturing the blight-resistance genes of Asian chestnuts.

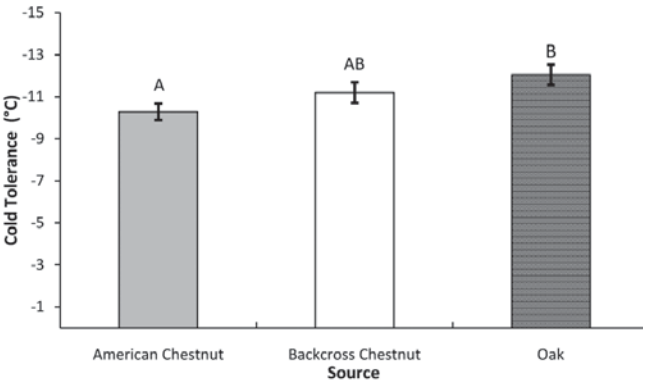


**Figure 1. Differences in mean (± SE) cold tolerance (*T*m) for American chestnut, backcross chestnut nuts, and northern red oak acorns ($n$ = 52). Means with different letters are significantly different ($p \leq 0.05$) based on ANOVA.**

In the northern part of its historic range, the species was most often found in lower-lying river valleys or locations influenced by larger lakes or the Atlantic Ocean (Harlow et al. 1979)—areas associated with more moderate microclimates. Although research into the cold hardiness of American chestnut is sparse, several sources indicate that it may be an important factor limiting American chestnut's competitive success in the north (Griffin and Elkins 1986, Gurney et al. forthcoming). Furthermore, because the Chinese chestnut used in backcross breeding may be particularly vulnerable to freezing injury (Jones et al. 1980), genetic mixes containing Chinese chestnut genes could have hardiness levels even lower than pure American stock.

An issue of particular concern to species restoration in cold climates is nut survival for natural reproduction. In general, the reproductive organs of plants are the tissues most sensitive to cold injury (Sakai et al. 1981), and the early literature suggests that the nuts of American chestnut may have been particularly susceptible to freezing damage (Paillet 2002). Because American chestnut has a large, unprotected nut, the cold hardiness of this tissue may limit restoration in cold northern regions.

To determine the potential for American chestnut restoration to the Northeast, we conducted a study comparing the cold tolerance of nuts of American chestnut, backcross chestnut, and northern red oak (*Quercus rubra*), a potential native competitor. The existence of genetic variation or local adaptation for nut cold hardiness within pure American chestnuts, as well as the potential influence of Asian chestnut genes, could be important considerations for future breeding.

Chestnuts from open-pollinated native trees in Maine, New York, Pennsylvania, Maryland, Virginia, Kentucky, and Tennessee, as well as nuts from backcross chestnuts in Vermont and Connecticut, were collected in the fall of 2006 by The American Chestnut Foundation staff and volunteers. Acorns from northern red oaks in Vermont were also collected in the fall of 2006 by Forest Service

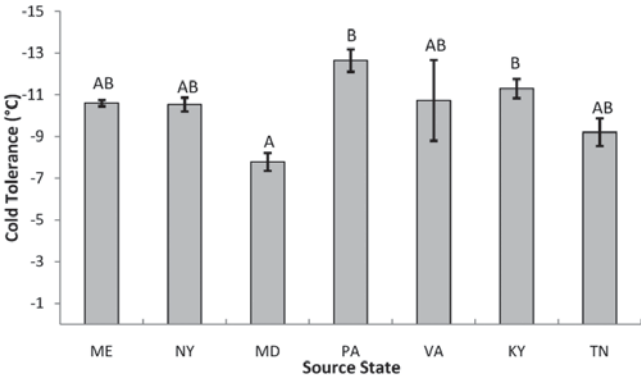


**Figure 2. Differences in mean (± SE) cold tolerance ($T_m$) of nuts from seven American chestnut sources ($n$ = 24). Means with different letters are significantly different ($p \leq 0.05$) based on results of a Tukey HSD test.**

staff. Chestnuts and acorns from Vermont were stored at 2–3°C in Burlington from time of harvest. Those collected outside Vermont were refrigerated near the site of collection, then shipped to Burlington in January and February of 2007 and stored together for a minimum of one week at 2–3°C to standardize environmental preconditioning prior to the experiment.

For cold tolerance tests, three to four nuts per source were first rinsed in distilled water, and then the pericarps, seed coats, hypocotyls, and radicles were removed. The remaining cotyledon material was cut into 5 mm cubes. Subsamples of two 5 mm cubes per nut were placed into individual cells of a 64-cell styrene tray for freezing. These duplicates within each tray were used to calculate mean electrical conductivity used in later curve-fitting analyses. Freezing stress was imposed using well-established methods (Schaberg et al. 2005). We selected 11 test temperatures, ranging from 5°C (no freezing stress) to -40°C (inducing complete mortality). Following exposure to a particular test temperature, injury was quantified as electrolyte leakage from sample tissues measured with a conductivity bridge. Initial conductivity was measured with a multielectrode instrument (Wavefront Technology, Ann Arbor MI), then samples were dried for more than 48 h at 40°C to kill the tissue, and the final conductivity was measured. Relative electrolyte leakage (REL), a measure of cell injury, was calculated as the proportion of initial conductivity of samples following damage at each subfreezing test temperature relative to the final conductivity of fully killed, oven-dried tissue. Then a sigmoid curve fitted to REL data for all test temperatures was used to calculate $T_m$, the temperature at the curve's midpoint as a measure of nut cold tolerance (Schaberg et al. 2005).

To test if cold tolerance estimates based on controlled freezing and electrolyte leakage measurements of cotyledon material reflected the influence of freezing on the germinative capacity of whole nuts, 110 nuts from New York were subjected to freezing temperatures (10 at each of the same 11 test temperatures), and the percent germination was measured after five weeks. Results of this evaluation showed that mean cold tolerance (-10.71°C) estimated from electrolyte leakage assays was within 0.6°C of mean cold tolerance estimated from germination tests (-11.27°C). The close association of these two estimates shows that electrolyte leakage of cotyledon tissue is an appropriate surrogate for cold tolerance measurements of intact nuts and suggests that the seed coat (removed for electrolyte leakage tests) provides little insulative protection to nuts.

An analysis of variance (ANOVA) of $T_m$ data identified a significant difference in cold tolerance among American chestnut, backcross chestnut, and red oak ($p$ = 0.0274); red oak (mean $T_m$ = -12.05°C) was approximately 2°C more cold tolerant than American chestnut (-10.28°C), with backcross chestnut (-11.20°C) being intermediate and statistically indistinguishable from the other two species (Figure 1). The overall difference between species, while significant, was minimal. Furthermore, it appears that the cold tolerance of the nuts from backcross chestnuts presents no disadvantage for natural regeneration relative to pure chestnuts. However, a more comprehensive analysis that includes more nuts and sources is needed to verify these preliminary findings.

An ANOVA of pure American chestnuts identified a significant difference in cold tolerance among sources ($p$ = 0.0095, Figure 2). A Tukey HSD test indicated that source differences were driven by the extremes in cold tolerance represented by the Maryland source (mean $T_m$ of -7.79°C), and the Kentucky (-11.30°C) and Pennsylvania (-12.64°C) sources. The other sources were intermediate and statistically indistinguishable in cold hardiness (Figure 2). These findings suggest the existence of genetic variation in nut cold tolerance within existing American chestnut populations, and thus the possibility of positive selection for nut cold tolerance within a breeding program. Correlation analyses detected no relationships between nut cold tolerance and the latitude ($p$ = 0.7081, $n$ = 7) or elevation ($p$ = 0.3763, $n$ = 7) of sources. This lack of geographic association could be due to genetic influences (e.g., founder effects, genetic drift, or inbreeding following the steep population declines) or could reflect environmental differences at mother tree sites that could have modified seed nutrition and physiology, thereby influencing measured cold tolerance. The confounding influence of environment on nut cold tolerance can best be controlled when mother trees are grown in a common garden (a future analysis).

The nuts of American and backcross chestnut were only minimally different or indistinguishable in cold tolerance from northern red oak acorns, suggesting that chestnuts are likely marginally, but adequately, cold tolerant enough for regeneration and establishment of the species in the Northeast under current climate conditions. Considering the limited hardiness of all sources tested, nut survival over winter likely relies on burial by rodents or the insulative

protection of snow cover to buffer nuts from damaging ambient conditions. While climate change predictions for the northeast include a general warming trend, future predicted winter temperature lows (Barron 2001) are well below our estimates of nut cold tolerance (Figure 1). Furthermore, predictions of decreased snow pack and increased soil freezing (Federer 2001) raise uncertainties about future overwintering nut survival of American chestnut.


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