Using Biotechnology to Help Restore the American Chestnut

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Abstract: American chestnut (*Castanea dentata*) once dominated the Appalachian forests of the Eastern United States, where it was a major timber and nut-producing tree. Beginning in the early 1900s, the tree was devastated by the chestnut blight fungus (*Cryphonectria parasitica*), which was accidentally introduced from Asia. As part of an effort to restore the species to the forest, we have been working with embryogenic cultures of the species, aiming to establish a reliable somatic embryogenesis system for mass clonal propagation, as well as for genetic transformation with potential anti-fungal genes. Our research over the past three years has overcome a number of bottlenecks in somatic embryo production, maturation, germination and conversion. We tested the effects of culture regime (semi-solid versus liquid), cold treatment, activated charcoal and somatic embryo morphology (i.e. cotyledon number) on germination and conversion of the somatic embryos. Cold treatment for 12 weeks was critical for conversion of chestnut somatic embryos to somatic seedlings, raising conversion frequencies for one line to 47%, compared to 7% with no cold treatment. Activated charcoal improved germination and conversion frequency for one line to 77% and 59%, respectively, and kept roots from darkening. For two lines that produced embryos with one, two or three-plus cotyledons, cotyledon number did not affect germination or conversion frequency. We also established embryogenic American chestnut suspension cultures and adapted a fractionation/plating system that allowed us to produce populations of relatively synchronous somatic embryos for multiple lines. Embryos derived from suspension cultures of two lines tested had higher conversion frequencies than those from cultures maintained on semi-solid medium. The improvements in manipulation of American chestnut embryogenic cultures described in this study have allowed over a 100-fold increase in somatic seedling production efficiency.

Keywords: American chestnut, Castanea dentata, somatic embryogenesis, germination, conversion, chestnut blight

American chestnut (*Castanea dentata*) was once the dominant forest species of the Appalachian Mountains in the eastern United States, with a range that extended from New England to Alabama. At the beginning of the 20th century, American chestnut contributed substantially to many local economies. The highly decay-resistant wood had many uses, including lumber for construction, shingles, furniture and fuel. High in tannin, chestnut was used for telephone poles, mine props, fences and in the leather industry. An annual crop of nuts made chestnut a major source of nutrition for man and wildlife. In the early 1900s, the species was almost eliminated as a forest tree due to the accidental introduction of the fungus *Cryphonectria parasitica*, which became known as the chestnut blight fungus. The blight is estimated to have killed around 3.5 billion trees (Roane et al. 1986). Since root systems are not infected by the fungus, American chestnut has escaped extinction because of its ability to sprout profusely and repeatedly from

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stumps and roots. Today, most American chestnut trees are found as shrubs or small trees in the forest. As part of an effort to restore the species to the forest, we have been working with embryogenic cultures of the species, aiming to establish a reliable somatic embryogenesis system for mass clonal propagation, as well as for genetic transformation with potential anti-fungal genes to develop transgenic trees that would be resistant to chestnut blight fungus.

MATERIALS AND METHODS

Culture initiation and maintenance and somatic embryo production

Immature chestnut burs, collected during summer 2001 and 2002, were provided by The American Chestnut Foundation (TACF) and the American Chestnut Cooperators Foundation (ACCF). Immature, open-pollinated seeds were used as explants. Using previously published protocols (Merkle et al. 1991, Carraway and Merkle 1997), embryogenic cultures were initiated by culturing the immature seeds on Induction/Maintenance Medium (IMM), which was a modified Woody Plant Medium (WPM; Lloyd and McCown 1980) supplemented with 0.5 g/l L-glutamine and 2 mg/l 2,4-D. After 3 months, clusters of repetitive embryos or proembryogenic masses (PEMs) were obtained. For continued culture proliferation clusters of repetitive embryos or PEMs were either transferred to fresh IMM every three weeks. After three to four weeks, 2-4 mm long embryos were selected using a dissecting microscope and transferred to 100 x 15 mm plastic Petri plates of semi-solid Embryo Development Medium (EDM), which was WPM lacking plant growth regulators. Twenty – 25 embryos were cultured per plate in a grid pattern with approximately 0.5 cm spacing. Embryos, which were oriented horizontally on the medium, were allowed to grow for 1 week prior to being subjected to the experimental treatments described below. All cultures were incubated in the dark at approximately 25° C.

Germination and conversion experiments

The cold treatment experiment used three lines. Embryos in this experiment were derived from PEMs that had been cultured on semi-solid IMM. Plates of embryos were divided into 3 groups to be given 0, 6 or 12 weeks of cold treatment. For the 0 weeks cold treatment, embryos from the first set of plates were transferred to GA-7 vessels (Magenta Corp.) containing 80 ml of semi-solid GM, which was the same as EDM, but lacking L-glutamine. Embryos were oriented with their radicle ends inserted into the gelled medium. GA-7s were placed in an incubator under cool white fluorescent light (100 $\mu mol \cdot m^{-2} \cdot s^{-1}$) with a 16 h photoperiod at 23° C. The remaining plates were wrapped in aluminum foil and placed in a dark refrigerator at 4° C. The second and third sets of plates were removed from the cold after 6 and 12 weeks, respectively, and embryos were transferred to GA7s and placed in the incubator under the same conditions described above. The numbers of germinated (i.e. showing radicle elongation) and converted (producing both roots and shoots) embryos were scored after six weeks in the incubator. The experiment was conducted twice with the same lines and run again with another line six months following the first experiment, in the last case using embryos derived from PEMs collected from size-fractionated suspension cultures.

The AC experiment used 4 lines and embryos were produced using the same protocol described for the first cold treatment experiment. However, in this case, harvested embryos were

transferred to Petri plates containing semi-solid EDM with or without 5 g/l activated charcoal (EM Industries) and allowed to grow for one week prior to storage in the refrigerator for 12 weeks at 4° C. Then, they were transferred to GA-7s containing 80 ml GM with or without 5 g/l AC (depending on whether they were matured on EDM with our without AC) and placed in the same incubator under the same conditions used for the cold treatment experiment. Germination and conversion frequencies were scored after six weeks. The entire experiment was not repeated in time with the same lines, but was repeated with another line three additional times, beginning six months later, with the additional treatment of 1 g/l AC. Embryos in these later replications were derived from size-fractionated suspension cultures.

In separate experiments from those described above, we compared the germination and conversion performances of somatic embryos produced from cultures maintained on gelled medium to those of embryos derived from size-fractionated suspension cultures of the same embryogenic lines. The first experiment used embryos from two lines, while the second experiment, conducted six months later, used embryos from an additional line. In both experiments, embryos were selected from populations derived either from PEMs grown on semi-solid medium or from size-fractionated and plated suspension cultures, and allowed to grow for one week. Then, plates were stored at 4° C for 12 weeks, after which embryos were transferred to GA-7s containing GM with 5 g/l AC and placed in the incubator. Six weeks later, numbers of germinated and converted embryos were scored.

We noticed that most of our lines produced embryos with varying morphology, most notably variation in cotyledon number. Since the embryos with either more or less than two cotyledons appeared otherwise normal, we speculated that these embryos might be capable of producing normal plantlets. Two lines that produced approximately equal proportions of embryos with one, two or three-plus cotyledons were chosen for an experiment to test the effect of this aspect of embryo morphology on germination and conversion. The experiment was conducted twice with one line and once with the other, using embryos that developed from size-fractionated and plated suspension cultures. Embryos were stored at 4° C for 12 weeks, transferred to GA-7s containing GM with 5 g/l AC and placed in the incubator. Six weeks later, numbers of converted embryos were scored.

RESULTS AND DISCUSSION

Cold treatment

Both line and cold treatment affected germination frequency ($P < 0.001$ and $P < 0.007$, respectively), although only cold treatment affected conversion frequency ($P < 0.001$). While some embryos germinated with no cold treatment, none of these completed the conversion process. Six weeks of cold failed to significantly improve germination or conversion frequency over no cold treatment, but 12 weeks of cold improved both, increasing average germination for the two lines from 34% to 51% and average conversion for these two lines from 0% to 12%. When the experiment was repeated with another line with embryos derived from PEMs collected from size-fractionated suspension cultures, cold treatment significantly ($P < 0.0014$) affected only conversion frequency, and again, only 12 weeks of cold increased it over the no cold treatment, in this case from 7% to 47%. We intend to test if even longer periods of cold treatment will further increase conversion frequency.

Activated charcoal

Since our chestnut cultures must be continuously cultured in medium with 2,4-D to maintain repetitive embryogenesis, we thought that AC might aid normal embryo development and maturation by adsorbing residual 2,4-D from them. In our first experiment with embryos of four culture lines produced on semi-solid medium, chi-square analysis indicated that the addition of 5 g/l AC did not significantly improve either germination frequency or conversion frequency. However, this result may be due to the fact that while AC improved germination and conversion for some clones, others actually had lower germination and conversion on medium with AC. When the experiment was repeated three times with somatic embryos derived from suspension cultures of another line in medium supplemented with 0, 1 or 5 g/l AC, both germination frequency and conversion frequency were improved by the addition of activated charcoal ($P < 0.001$). Although 1% AC gave the highest germination (77%) and conversion (59%) frequencies, these were not statistically higher than for the 5 g/l AC treatment. The different results with regard to the impact of AC on germination and conversion of embryos from the first experiment with four related lines and the second set of experiments with one line unrelated to the first set may be attributable to genotypic differences. However, it is also possible that embryos from the second set of experiments benefited more from the AC due to the fact that they were derived from suspension cultures, in which the PEMs were bathed continuously in 2,4-D-supplemented medium, rather than sitting on top of gelled medium with 2,4-D.

One qualitative difference we noted in all the AC experiments was that roots of somatic seedlings in the AC medium remained white, while those in medium lacking AC darkened during the 6 weeks the somatic seedlings remained in the GA-7s. Thus, it is possible that one or more of the potential benefits of AC noted by Pan and van Staden (1998), the establishment of a darkened environment or adsorption of undesirable substances, played a role in keeping the roots from browning. In addition, somatic seedlings growing in medium with AC appeared to have a greater tendency to generate branching root systems than those in medium without AC, although this variable was not measured. Given these differences and that our standard embryo production procedure currently uses suspension cultures, we now routinely germinate chestnut somatic embryos on medium with 5 g/l AC.

Culture regime

Embryos derived from suspension cultures converted at a higher frequency than those derived from cultures maintained on semi-solid medium for two of the three tested lines. The first experiment comparing germination and conversion of embryos derived from semi-solid versus liquid medium used two lines. Chi-square analysis of combined data from these two lines indicated that culture regime failed to affect conversion frequency ($P<0.09$). However, when the two clones were analyzed separately, chi-square results indicated that culture regime improved conversion for somatic embryos of one line, but had no significant effect on embryos from the other line ($P<0.08$). In the second experiment, which tested a single line, embryos derived from suspension cultures converted at a significantly ($P <0.001$) higher rate than those derived from material cultured on semi-solid medium. Somatic embryos arising from the size-fractionated suspensions tended to be singularized or in loose clusters compared to the fused clusters of embryos produced on semi-solid medium. Thus, there was probably less damage, particularly to

the radicles, when individual embryos from the liquid suspension were harvested for germination. While the superiority of suspension culture-derived embryos did not hold for every line in these two experiments, we have adopted size-fractionation and plating of suspension cultures as our standard method of embryo production for most of our embryogenic American chestnut lines.

Embryo morphology

Malformed somatic embryos are often reported in somatic embryogenesis studies. No doubt these are often discarded as being unlikely to produce viable plants. We noted high percentages of embryos with single cotyledons or more than two cotyledons in our cultures, but these embryos appeared similar in other respects to those with two cotyledons, with a well-defined shoot apical meristem and radicle. Therefore, we divided populations of embryos from two lines into those with one, two, or three or more cotyledons, put them through our standard germination treatment (12 weeks of cold, culture on EDM with 5 g/l AC) and measured conversion. Overall, the three cotyledon classes did not differ in their conversion frequencies ($P<0.06$), although embryos from on line with a single cotyledon appeared to have a lower conversion than the other two classes. Based on these results, we feel that we can safely select embryos with two or more cotyledons for somatic seedling production, and in for some clones, even embryos with only a single cotyledon will produce plantlets.

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LITERATURE CITED

Carraway, D.T., and S.A. Merkle. 1997. Plantlet regeneration from somatic embryos of American chestnut. Can. J. For. Res. 27:1805-1812.

Lloyd, G., and B. McCown. 1980. Commercially feasible micropropagation of mountain laurel; *Kalmia latifolia*; by use of shoot-tip culture. Proc. Int. Plant Propag. Soc. 30:421-427.

Merkle, S.A., A.T. Wiecko, and B.A. Watson-Pauley. 1991. Somatic embryogenesis in American chestnut. Can. J. For. Res. 21:1698-1701.

Pan MJ, van Staden J (1998) The use of charcoal in *in vitro* culture – a review. Plant Growth Regulation 26:155-163

Roane M., Griffin G. and Elkins J.R, 1986. Chestnut blight. In: Chestnut Blight, Other Endothia Diseases and the Genus *Endothia*, APS Monograph Series, APS Press, St. Paul, MN, USA, pp. 1-26.

Total Inside-Bark Volume Estimation for Loblolly Pine (*Pinus taeda* L.) in Genetic Trials.

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Many tree breeding programs select genotypes based on volume more than any other trait. These programs commonly estimate volume using a combined-variable equation that uses diameter at breast height (D) squared multiplied by total tree height (H) as a regressor on inside-bark volume. The North Carolina State University–Industry Cooperative Tree Improvement Program (TIP) has a long history using the Warner and Goebel (1963) combined-variable equation (Warner-Goebel equation). The Warner-Goebel equation was derived from 74 loblolly pine trees from the upper South Carolina Piedmont with least-squares regression. Combined-variable equations have been effective in fitting prediction equations for total inside-bark volume to small data sets (Spurr 1952). However, differences in taper and bark thickness among individuals or families are not accounted for with a combined-variable equation. The objective of this abstract is to evaluate selection with the Warner-Goebel equation.

METHODS AND MATERIALS

A genotype by cultural treatment study at Bainbridge, GA was measured in the 13th growing season. This study was a two by two factorial of weed control and fertilizer treatments in a split-plot design. For the main cultural treatment plots, the control had no herbicide applications until age five and no fertilizer treatments. The herbicide treatment consisted of early woody and herbaceous competition control and all treatments were aerially released at age five. The fertilization treatment was ground applied five times up until age 9.

There were 25 open-pollinated first- and second-generation families arranged as individual-tree subplots. Each complete block was replicated 5 times. Some known relationships existed between mothers and were acknowledged in the estimation of genetic parameters. Approximately 40 individuals from each of 25 families were sampled, 10 from each treatment. Two trees from each family were sampled in most replication/treatment plots.

Sectional data were collected along the stem every 1.2 m to a 7.6-cm top. Inside-bark diameters were used in Smalian's log volume equation (Avery and Burkhart 2002) to find the inside-bark volume of each stem section. In addition to diameters, total height and height to live crown were measured to calculate crown ratio as a percentage of the total height.

Volumes estimated from the Warner-Goebel equation were compared to those determined by stem analysis of the felled tress. The metric form of the Warner-Goebel (1963) equation was

$$\hat{V}_{ib} = 0.954560899 + 1.092111257\left(\frac{D^2H}{10}\right)$$

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where $\hat{V}_{ib}$ is the estimated total inside-bark volume in dm^3 and D^2H is the combined variable in cm^2 x m. Selection efficiency was calculated using methods from Falconer and MacKay (1996). The correlated response was

$$CR_y = ih_x h_y r_A \sigma_{py}$$

where CR_y is the correlated response of indirect selection for trait y, i is the selection intensity, h_x is $\sqrt{\text{heritability of trait } x}$, h_y is $\sqrt{\text{heritability of trait } y}$, r_A is the genetic correlation between traits x and y, and σ_{py} is the phenotypic standard deviation of trait *y*.

The response to direct selection on trait *y* was:

$$R_y = ih_y^2 \sigma_{py}$$

where R_y is the response from direct selection and h_y^2 is the heritability of trait *y*. Selection efficiency was calculated as:

$$S = \frac{CR_y}{R_y}$$

RESULTS AND DISCUSSION

The family-mean heritabilities of the measured volume from stem section data and the estimated volume using the Warner-Goebel equation were 0.69 and 0.72, respectively. The standard errors for these two values were 0.10 and 0.09, respectively. The genetic correlation between these two traits was high (0.99 with a standard error of 0.0056). Consequently, the selection efficiency for selection of estimated total inside-bark volume to make gains in measured inside-bark volume was 1.01. Comparison of the total inside-bark volume estimates from the Warner-Goebel equation and from the destructive sample can be seen in Figure 1. The Warner-Goebel equation generally over-estimated the total inside-bark volumes measured in the field. However, this did not affect selection.

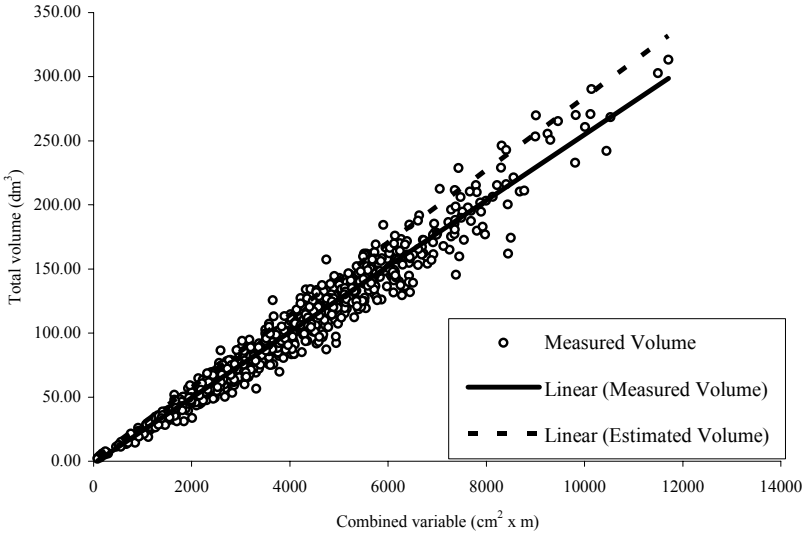

Figure 1. Comparison of prediction lines for estimated volume from the Warner-Goebel equation and measured inside-bark volume.

Combined-variable equations are commonly used to estimate volume in many research applications. For TIP trials, individual stems are generally compared using the Warner-Goebel total inside-bark volume prediction equation. Selection for true inside-bark volume by estimating volume with this equation has been an effective practice based on selection efficiency. In fact, family selection for volume estimated by prediction equations was slightly more favorable than by destructively sampling. This high selection efficiency may have resulted from less error variance being associated with estimated volume than with the detailed volume measurement. The measured volume may have had more error variance due to variation in stem taper, bark thickness, environmental variation, and unknown factors. Nonetheless, D and H measurements capture a great deal of the genetic variation in total inside-bark volume. Differences in volume not accounted for by estimating volume with the combined-variable equation are probably small enough for indirect selection to be effective. It is recommended to continue using a combined-variable equation for selection of superior loblolly pine genotypes in the Southeastern US.

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REFERENCES

Avery, T. E. and H. E. Burkhart. 2002. Forest Measurements. Fifth Ed. McGraw-Hill. New York. 456 p.

Falconer, D. S. and T. F. C. MacKay. 1996. Introduction to Quantitative Genetics. 4th Edition Ed. Prentice Hall. Essex, England. 464 p.

Foster, G. S. 1988. Growth and morphology of rooted cuttings and seedlings of loblolly pine and their genetic analysis. P. 67-78 *in* Proceeding , 10th North American Forest Biology Workshop.

Pederick, L. A. 1970. Variation and inheritance of stem form and bark thickness in young loblolly pine. Technical Report 41 North Carolina State University. Raleigh, NC.

Spurr, S. H. 1952. Forest Inventory. Ed. Ronald Press Co. New York. 476 p.

Stelzer, H. E., G. S. Foster, V. Shaw and J. B. McRae. 1998. Ten-year growth comparison between rooted cuttings and seedlings of loblolly pine. Canadian Journal of Forest Research. 28: 69-73.

Warner, J. R. and N. B. Goebel. 1963. Total and bark volume tables for small diameter loblolly, shortleaf, and Virginia pine in the upper South Carolina piedmont. Forest Research Series No. 9. Clemson University. Clemson, South Carolina.