Advances in American Chestnut Somatic Seedling Production

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Abstract: The American chestnut was one of the most important forest trees in the Appalachian Forest until the introduction of the chestnut blight fungus, which caused the death of virtually every mature American chestnut tree in the eastern United States. A system for mass propagation of blight-resistant material obtained through conventional breeding or gene transfer is still lacking. Thus, the goal of our project is to develop a high-frequency in vitro propagation system for American chestnut via somatic embryogenesis. Two bottlenecks in this approach are the low initiation rate of embryogenic cultures and the low production efficiency of plantlets (somatic seedlings) from the somatic embryos. To increase embryogenic culture initiation, we tested two plant growth regulators (2,4-D and picloram) at different concentrations and found that 2,4-D resulted in the highest frequency of embryogenesis (up to 3.5 %). This culture initiation experiment also demonstrated for the first time that highly productive embryogenic cultures could be initiated from immature seeds resulting from controlled crosses between known American chestnut parents. To increase plantlet production, we tested variations in cold (4° C) treatment duration (12, 15, and 18 weeks) and light quality (red, red + far red, and cool white fluorescent). For some genotypes, the longer cold treatments improved plantlet production and red light improved overall plantlet production frequency (up to 80% and 69%, respectively). Thus, by manipulating the cultural treatments, we were able to increase American chestnut somatic seedling production efficiency above the levels we previously reported. The first American chestnut somatic seedlings to be tested under nursery conditions were promising, growing up to 1.5 m in their first season. These advances in clonal propagation will aid in the restoration of the American chestnut to our forests.

INTRODUCTION

Prior to the early 20th century, American chestnut (*Castanea dentata*) was one of the most important forest trees in the United States, comprising up to a quarter of the trees in the southern Appalachian forest. The accidental introduction of the chestnut blight fungus, *Cryphonectria parasitica,* on Asian chestnut stock at the turn of the century resulted in the death of virtually every mature American chestnut tree from New England to Georgia within 40 years (Burnham 1988). Currently, the tree only exists in its natural environment as a minor component of eastern hardwood forests, surviving in the form of sprouts from old stumps and root systems, since the fungus does not penetrate the roots (Viéitez and Merkle 2005).

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While the blight remains an obstacle to the natural reestablishment of chestnut in our eastern forests, conventional breeding and gene transfer research currently underway should result in the production of blight-resistant American chestnut genotypes within the next 5-7 years. However, even once blight-resistant American chestnut genotypes become available, there exists no method for mass propagating the blight-resistant genotypes to meet the anticipated demand for planting stock. Somatic embryogenesis may offer the potential for mass clonal propagation of blight-resistant American chestnut genotypes, if somatic seedling production efficiency for those genotypes can be raised to useful levels.

Somatic embryogenesis in American chestnut (*C. dentata*) was first reported over a decade ago (Merkle et al. 1991). Embryogenic cultures were initiated at a low frequency (less than 1%) by culturing immature seeds on Woody Plant Medium (WPM; Lloyd and McCown 1980) with 2,4-dichlorophenoxyacetic acid (2,4-D), but no plantlets were produced. Carraway and Merkle (1997) found that initiation medium with indole-3-acetic acid (IAA) and 2,4-D induced an embryogenic response, but only cultures initiated on medium with 3 mg/L 2,4-D sustained production of somatic embryos for more than 2 months. In addition, for continued proliferation via repetitive embryogenesis, cultures had to be maintained continuously on medium with 2,4-D.

Plantlet production from American chestnut embryogenic cultures was problematic for several years. A 12-week cold (4º C) stratification period prior to germination tested by Carraway and Merkle (1997) improved development and germination of American chestnut somatic embryos, although conversion to plantlets was very rare, and none of the plantlets survived transfer to *ex vitro* conditions. Xing, et al. (1999) reported successful production of 20 American chestnut plantlets from somatic embryos without a cold treatment, but it was unclear how many of these plantlets were true somatic seedlings and how many embryos produced only shoots that had to be rooted in a separate step to produce complete plantlets. Robichaud et al. (2004) used a minimum of four weeks of cold (4º C) stratification and generated a few American chestnut somatic seedlings that survived and continued to grow following transfer to the greenhouse. Andrade and Merkle (2005) tested 0, 6, and 12-week durations for the cold (4º C) stratification period and found that 12 weeks of cold improved germination and conversion frequencies of American chestnut somatic embryos. While 12 weeks of cold stratification yielded the highest plantlet production frequencies and this treatment was incorporated into the standard protocol for plantlet production, longer cold treatments were not tested.

The standard protocol for American chestnut somatic embryo plantlet production employs a 16-h photoperiod under cool white fluorescent light (100 μmol m^{-2} s^{-1}) (Andrade and Merkle 2005). Red light improved germination and conversion frequencies for southern pine somatic embryos (Merkle et al. 2006). Kvaalen and Appelgren (1999) also reported that red light improved somatic embryo germination frequencies in Norway spruce (*Picea abies*), but did not improve germination of zygotic seedlings. Other light qualities have yet to be tested for their effects on chestnut somatic embryo germination and conversion.

The overall goal of the research reported here was to enhance our ability to generate embryogenic cultures of American chestnut and to produce plantlets from the cultures so that the system can ultimately be used for propagation of blight-resistant material from conventional breeding programs and for engineering with potential blight-resistance genes. The specific

objectives of the research were to: (1) Test different plant growth regulators and concentrations for their potential to improve the frequency with which new American chestnut embryogenic cultures can be initiated from immature seed explants, and (2) To test extended cold treatments and different light qualities for their potential to improve the germination of American chestnut somatic embryos and their conversion to somatic seedlings.

MATERIALS & METHODS

Culture Initiation Experiment

Immature American chestnut burs were collected from select mature trees, representing 11 families that were either open-pollinated or cross-pollinated with pollen from a known source tree. Altogether, burs were collected from eight source trees in Kentucky, Georgia, Connecticut, and Pennsylvania during late July and early August 2006. The burs were dissected to remove the nuts, which were surface-sterilized by agitation in the following solutions: 70% ethanol for 20 s; 10% Roccal (10% Alkyl dimethyl benzyl ammonium chloride, Winthrop Laboratories Div., Sterling Drug Co., New York, NY) for 1 min; 5.25% sodium hypochlorite for 5 min; sterile water for 3 min; 0.01N HCl for 3 min, and three subsequent rinses in sterile water for 3 min each. Then, the developing nuts were dissected to remove the immature seeds for culture. The number of seeds cultured for each family ranged from 45 to 994 (Table 1).

The immature seeds were cultured in 60x15-mm plastic Petri dishes on semi-solid initiation and maintenance medium (IMM; Andrade et al. 2005), modified with different auxins and concentrations. Two mg/l and 4 mg/l of both 2,4-dichlorophenoxyacetic acid (2,4-D) and 4-amino-3,5,6-trichloropicolinic acid (picloram) supplements were tested, for a total of four experimental plant growth regulator (PGR) treatments (2 mg/l 2,4-D for IMM1, 4 mg/l 2,4-D for IMM2, 2 mg/l picloram for IMM3, 4 mg/l picloram for IMM4). After autoclaving the medium and allowing it to cool, 0.5 mg l^{-1} L-glutamine was filter-sterilized and added.

All the live seeds from a given nut (ranging from 9 -18) were cultured together on a single Petri plate. The cultures were incubated in the dark at 25º C. Seed explants were transferred to fresh medium of the same composition after 5 weeks and the cultures were examined for signs of embryogenic proliferation after 10 weeks. The result for each seed explant was recorded, and the percentage of seeds that produced embryogenic material was calculated for each family and each PGR treatment. Those that appeared to be embryogenic after 10 weeks were maintained thereafter by subculture on fresh IMM1 or IMM3 every 3 weeks.

Somatic Embryo Germination and Conversion Experiments

Cultures used for somatic embryo germination and conversion experiments had been initiated in previous years from open-pollinated seeds collected from source trees in Virginia. Embryogenic clones WB569-37, WB569-61 and WB569-97 were initiated from seeds collected from one source tree in 2003, WB484-3 was initiated from a second tree in 2003, and AM54-1 and AM58-1 were initiated from a third tree in 2001. Suspensions were initiated from the American chestnut embryogenic cultures by inoculating approximately 0.5 g of proembryogenic masses (PEMs) into 125-ml Erlenmeyer flasks containing 30 ml of liquid IMM (with 0.5 mg l^{-1} L-glutamine). Suspensions were maintained by shaking on a gyratory shaker at 100 rpm at 25º C

in the dark. Cultures were fed every 2 weeks by aspirating out the old medium and adding 30 ml of fresh IMM.

After 45 days in suspension, the PEMs were collected by size-fractionating with stainless steel CELLECTOR sieves (Bellco Glass), based on the method described previously by Andrade et al. (2005). Suspensions were poured through nested sieves with pore sizes of 860 µm and 38 µm, such that cell clusters with diameters between the two pore sizes were collected on the 38 µm sieve. Using a pipette, the PEMs collected in the 38 µm sieve were backwashed from the sieve into a new, sterile 125 ml Erlenmeyer flask with 30 ml of liquid embryo development medium (EDM; Andrade et al. 2005). Re-suspended PEMs were incubated under the same conditions as described above for an additional 4 days. To plate the PEMs for embryo development, they were collected on Nitex nylon mesh with a 30 µm pore size (Sefar America, Depew, NY) using a Büchner funnel with mild vacuum. The mesh with PEMs was subsequently placed on semi-solid EDM in 60x15-mm plastic Petri dishes and incubated in the dark at 25º C.

Once the PEMs had developed into embryos reaching lengths of 2 to 4 mm, those possessing a morphology similar to that of zygotic embryos (i.e. with a radicle and two cotyledons) were selected using a dissecting microscope and transferred to semi-solid EDM in 100 x 15-mm plastic Petri plates. In cases where few embryos had the ideal form, those with a well-defined radicle and one to several "leafy" cotyledons were selected. Twenty embryos were placed in a grid pattern on each Petri plate. The embryos were oriented horizontally on the medium and were incubated at 25º C for 1 week in the dark prior to cold stratification treatments.

Cold treatment experiment

The effect of extended cold treatments on germination and conversion frequencies was tested by storing embryos from three embryogenic clones for 12, 15 or 18 weeks in a refrigerator at 4° C. The three embryogenic clones (WB569-37, WB569-61, and WB569-97) were derived from immature seeds of a single mother tree growing in Virginia, provided by TACF cooperators. Additional treatments to determine whether a transfer to fresh EDM during the cold treatment would improve germination and conversion frequencies were also tested. This variable was tested for only the 15-week and 18-week treatments. After 12 weeks of cold treatment, subsamples of the embryos in the 15- and 18-week treatments were transferred to plates of fresh EDM (Andrade and Merkle 2005) and returned to the refrigerator, while the other embryos remained on the same plate of EDM for the duration of the cold treatment. In total, there were 5 treatments, including 12-, 15-, and 18-week cold treatment duration treatments without transfer to fresh medium and 15- and 18-week cold treatments with transfer to fresh medium.

At the end of the cold stratification period, embryos were selected from the plates and transferred to GA7 vessels containing 100 ml of semi-solid GM (Andrade and Merkle 2005) with 5 g l^{-1} activated charcoal (EM Industries). Five embryos were placed in each GA7, oriented vertically (radicle down). Three GA7s (15 embryos total) were used for each genotype by treatment combination. GA7s with embryos were placed in an incubator at 25° C under cool white fluorescent light ($100 \cdot \mu mol \cdot m^{-2} \cdot s^{-1}$) with a 16-h photoperiod. Germination and conversion frequencies were tallied after 6 weeks of incubation.

Light quality experiment

To assess the effect of light quality on chestnut somatic embryo germination and conversion, different light qualities in the plantlet production stage were tested. The standard protocol was followed for embryo production for three embryogenic clones (AM58-1, AM54-1, and WB484-3), with the substitution of an 18-week cold duration instead of the standard 12 weeks. Using two Percival Model E-30LED incubators with light-emitting diodes (LEDs), we tested red light (600 nm) and red+far-red light (600 nm + 750 nm) wavelengths in comparison to our standard cool white fluorescent light ($100 \cdot \mu mol \cdot m^{-2} \cdot s^{-1}$) with 16-h photoperiod, for their effects on somatic embryo germination and conversion. We used three GA7s with 5 embryos each for a total of 15 embryos for each clone by light quality treatment combination. Germination and conversion frequencies were recorded after 6 weeks in the incubators.

Statistical analysis

Culture initiation data and somatic embryo germination and conversion data were analyzed by analysis of variance using PROC GLM and Duncan's Multiple Range Test in SAS (SAS Institute 1990).

RESULTS

Culture Initiation Experiment

Explants from 7 of the 11 families produced at least one embryogenic culture (Table 1). Of the 4,040 seeds that were placed in culture, only 97 produced embryogenic material, for an overall embryogenesis induction frequency of 2.4%. Embryogenesis induction frequency varied significantly among the successful families ($P<0.0001$) in the initiation experiment, with initiation frequencies ranging from 0.47% to 5.53% (Table 1). Two families with the same female parent (Adair x Kelly and Adair x Wayne3) gave significantly different initiation frequencies (5.53% and 2.00%, respectively), indicating a possible influence on embryogenesis due to the male parent.

Table 1. Embryogenesis induction results for seeds of 11 American chestnut families cultured in 2006.

Family	total # seeds	# embryogenic seeds	% embryogenesis
GAUNI3 x GAFAN2	211	1	0.47
GAUNI3 x OP	169	8	4.73
MY1 x ALA	541	4	0.74
RHR2T2	288	0	0.00
RHR2T7	45	1	2.22
RHR3T7	93	0	0.00
RHR4T3	126	0	0.00
Adair x Kelley	994	55	5.53
Adair x Wayne3	701	14	2.00
Stocker1 x Adair	414	0	0.00
McClosky x OP	458	14	3.06
OVERALL	*4040*	*97*	*2.40*

The 2 mg/l 2,4-D treatment yielded the highest average frequency (1.26%) of embryogenesis across the 7 families. However, Duncan's test indicated that this PGR treatment was only significantly superior to the 4 mg/l picloram treatment (0.24%) (Fig.1). While some families produced embryogenic material with all four PGR treatments, two families responded only to a single PGR treatment. Also, while the 2 mg/l 2,4-D treatment produced the highest average initiation rate among the five families that responded, the 2 mg/l picloram treatment produced a response from more families (six families). Both treatments that employed a PGR concentration of 4 mg/l produced embryogenic material in the same four families.

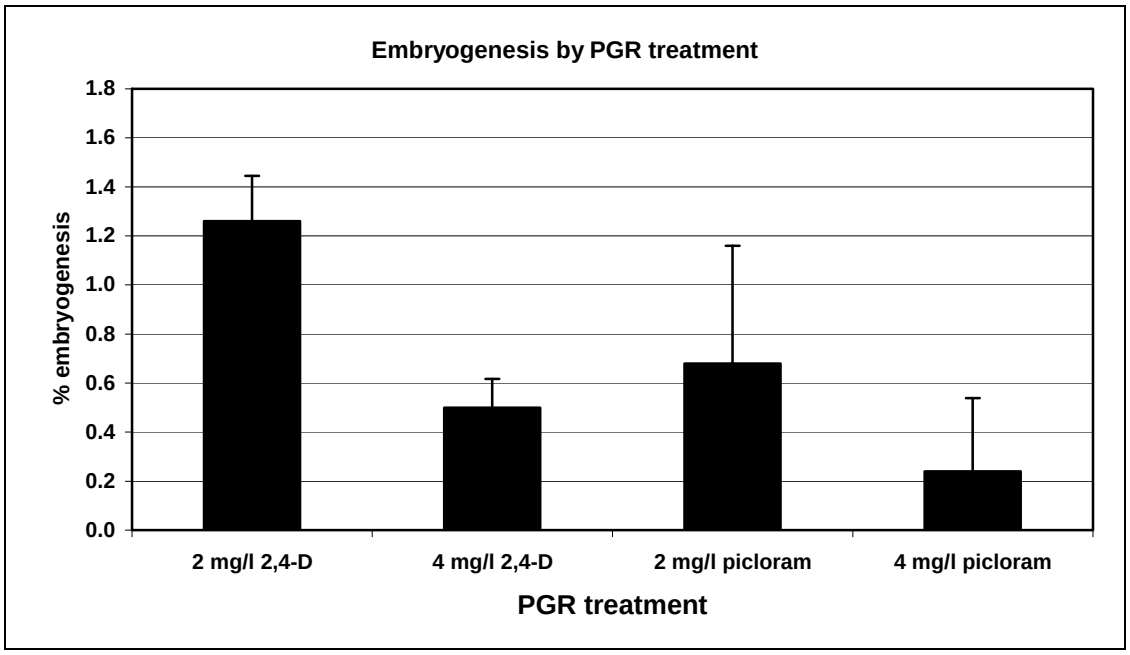

Figure 1. Effect of plant growth regulator treatments on embryogenesis induction from seeds of seven American chestnut families, averaged across all families. Bars indicate standard error.

Cold Treatment Experiment

Overall, analysis of variance indicated that both clone and clone x treatment interaction significantly affected germination frequency ($P<0.0001$), but only clone significantly affected conversion frequency ($P<0.0001$). However, according to the Duncan's test results, the 15-week with transfer and 18-week with transfer treatments yielded significantly higher germination rates (both 60.0%) than the 15-week treatment (44.4%). Duncan's test also indicated that the overall conversion rates were significantly different between the 18-week with transfer treatment (35.6%) and the 15-week treatment (15.6%) (Fig. 2).

When the results for each clone were examined individually, the nature of the interaction between clone and cold treatment on germination frequency became apparent, in that individual clones responded differently to the different cold treatments (data not shown). One clone (WB569-61) failed to germinate or convert to plantlets following the 15-week and 18-week cold

treatments without a transfer, but germinated and converted in the 15-week and 18-week cold treatments that included a transfer to fresh medium. This clone was consistently the lowest plantlet producer of the three clones across all cold treatments. A majority of the somatic embryos for this clone turned brown and stopped developing in the 15- and 18-week treatments that did not include a transfer to fresh medium.

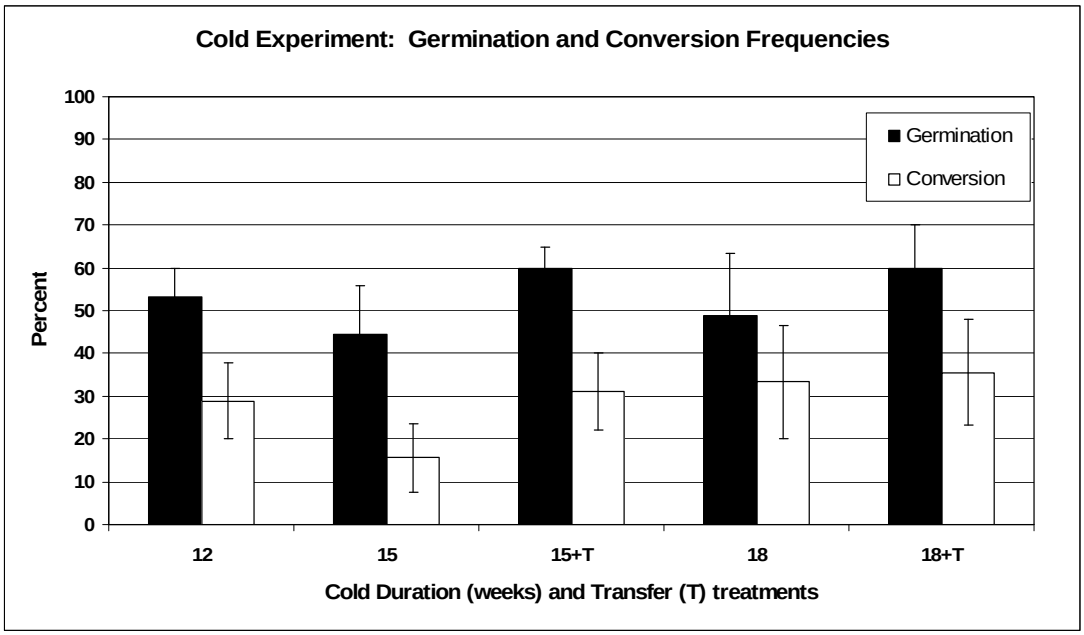

Figure 2. Results for the effect of cold and transfer treatments on average germination and conversion of American chestnut somatic embryos for three clones. Bars indicate standard error.

For clone WB569-97, there were no significant differences among the cold treatments for either germination or conversion. Embryos of this clone developed faster in the cold treatments that did not include a transfer, as indicated by greater radicle elongation by the somatic embryos that were not transferred compared to very little radicle elongation by those that were transferred to fresh medium. However, quantitative data for this observation were not recorded. For clone WB569-37, the 18-week and 18-week with transfer treatments yielded significantly higher germination frequencies (100% and 93.3%, respectively) than the 12-, 15-, and 15-week with transfer treatments (66.7%, 66.7% and 73.3%, respectively), according to Duncan's test. For this clone, the 18-week and 18-week with transfer treatments also yielded significantly higher conversion frequencies (80.0% for both treatments) than the 15-week treatment (33.3%).

Light Quality Experiment

Light quality treatment, clone, and clone x light quality treatment combination all had significant effects on germination frequency ($P = 0.0039$, $P = 0.0282$, and $P = 0.0069$, respectively). The light quality treatment also had a significant effect on conversion frequency ($P = 0.0022$). Duncan's test indicated that both germination and conversion frequencies were significantly higher under the red and red + far-red treatments (R germ: 86.7%, R+FR germ: 73.3%, R conv:

68.9%, R+FR conv: 57.8%), compared to the white light control (germ: 48.9%, conv: 31.1%) (Fig. 3).

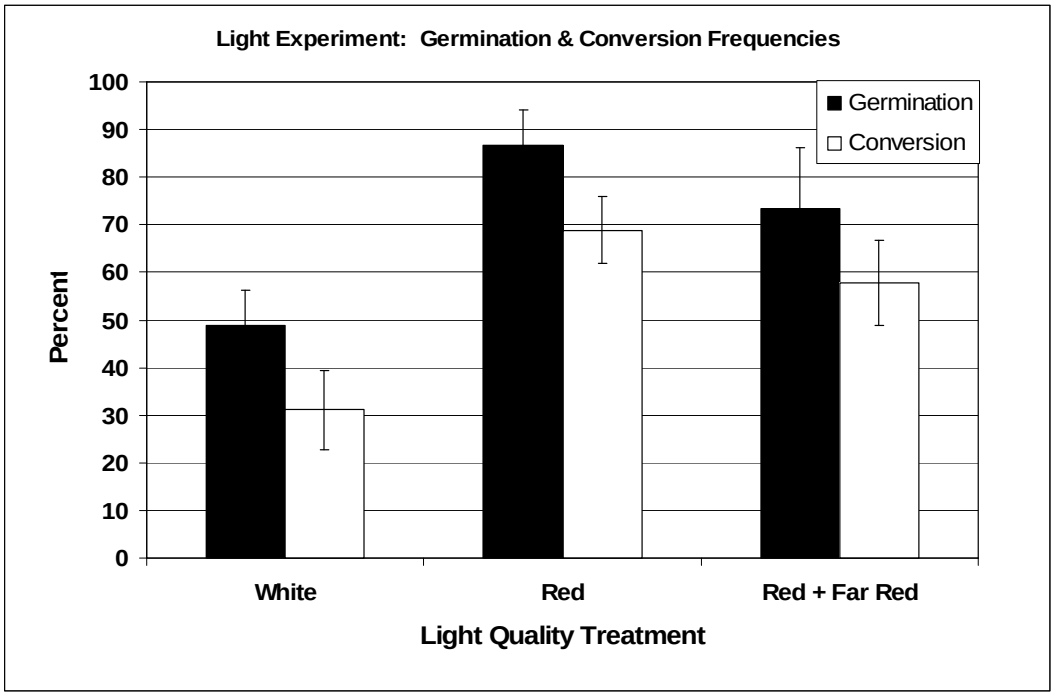

Figure 3. Effects of light quality treatment on germination and conversion of American chestnut somatic embryos, averaged across three clones. Bars indicate standard error.

As was the case with the cold treatment experiment, when the results for each clone were examined independently, the differential responses among the clones to light quality treatment became apparent (data not shown). In two of the three clones, red light significantly increased plantlet production. Light quality was especially critical for clone AM58-1. For this clone, Duncan's test indicated that germination ($P = 0.0016$) and conversion ($P = 0.0115$) frequencies were significantly higher for both the red and the red + far-red treatments than for the white light treatment. This difference is due to the fact that somatic embryos of AM58-1 completely failed to germinate and convert to plantlets under white light, but successfully produced somatic seedlings under red and red + far-red light, with germination rates of 86.7% and 80.0%, respectively, and conversion rates of 73.3% and 60.0%, respectively. For clone WB484-3, conversion frequency was significantly enhanced by red light (86.7%) compared to white light (53.3%), according to Duncan's test. For clone AM54-1, however, there were no significant differences in germination or conversion frequencies among the different light quality treatments. Somatic embryos also seemed to germinate more rapidly under red and red + far-red light compared to white light; however, these were only visual observations and no quantitative data were recorded.

DISCUSSION

Results from the experiment to test different PGRs and concentrations for their effect on embryogenesis induction indicated that each family reacted differently to each of the four PGR treatments. This is likely due to the genetic variation among the different families tested. Another possible explanation is that the seeds from the different mother trees may have been in different stages of development inside the nuts when they were harvested from the trees, and therefore may have reacted differently to the treatments. Unfortunately, it is impossible to schedule the harvest precisely for each bur on each tree and therefore, this is one source of uncontrollable variation. However, in general, 2 mg/l of either 2,4-D or picloram gave higher induction frequencies than 4 mg/l of either PGR. Based on these results, we concluded that there is no justification for replacing our standard 2 mg/l 2,4-D treatment for culture initiation with one of the alternative induction treatments. These results are similar to those of previous reports in which initiation medium with indole-3-acetic acid (IAA) and 2,4-D induced an embryogenic response, but only cultures initiated on medium with 3 mg/L 2,4-D sustained production of somatic embryos for more than 2 months (Carraway and Merkle 1997). It is possible that factors other than those that can be manipulated during the culture process (e.g. genotypic control over the embryogenesis response, low percentage of fertilized seeds) control embryogenesis induction in American chestnut, such that initiation frequencies will remain low regardless of what plant growth regulators or other culture variables are tested.

The results of the cold treatment experiment did not indicate that a cold treatment longer than 12 weeks would significantly improve germination and conversion frequencies for all clones of American chestnut that we tested. However, for some clones, a longer cold treatment (18 weeks) increased germination and conversion frequencies. If a longer cold treatment is needed, our results indicate that a transfer to fresh medium following the first 12 weeks of cold may produce higher rates of plantlet production. The variation among clones in response to the different cold treatments is likely attributable to genetic variation. We concluded that the optimal cold treatment duration will need to be determined for each American chestnut clone to maximize plantlet production efficiency. These conclusions agree with previous observations that various clones respond differently to a range of cold treatment durations (Andrade and Merkle 2005).

As with the cold treatment experiment, there was considerable variation in the responses of different lines to the different light quality treatments, which is again likely attributable to genetic variation among the lines. Overall, red light and red + far-red light improved germination and conversion rates compared to white light, and red light was superior to red + far-red light. The positive effects of red light on germination and conversion of somatic embryos have also been reported for southern pine species (Merkle et al. 2006) and Norway spruce (Kvaalen and Appelgren 1999). We concluded that somatic embryo germination under LED-supplied red light in a 16-h photoperiod at 25º C should be incorporated into our American chestnut plantlet production protocol.

While we were unable to improve culture initiation frequencies by manipulating plant growth regulator type or concentration, our results indicate that further enhancement of American

chestnut somatic seedling production efficiency is possible by manipulating cultural treatments such as cold stratification and light quality during the periods prior to and during germination. The ability to produce more plants from American chestnut cultures will be especially useful for propagation of trees from genetically transformed cultures, as recently reported by Polin et al. (2005) and Andrade et al. (2006). While plantlet production efficiencies still need to be greatly improved, we believe these enhancements will help move our embryogenesis system from a laboratory phenomenon towards a useful propagation system for blight-resistant American chestnut trees. Of course, the ability of these trees to grow in the field has yet to be determined. But the first American chestnut somatic seedlings from our research program to be planted in the nursery (following one year in the greenhouse) performed well in their first season, with some reaching heights over 1.5 m. A combination of plantlet production protocol improvements and genetic enhancement of fungal resistance in American chestnut will allow us to make substantial contributions to the restoration of this species to our forests.

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