PRESERVATION AND MULTIPLICATION OF ELITE BACKCROSS AMERICAN CHESTNUT HYBRIDS BY MICROPROPAGATION

A report prepared for The American Chestnut Foundation
Submitter November 2, 2013

Summary

One of the bottlenecks for The American Chestnut Foundation's breeding program is the inability to clonally propagate elite lines from B3F3 trees demonstrating the highest levels of blight resistance. We attempted to adapt the protocols we have developed for tissue culture propagation of transgenic American chestnut clones to the B3F3 trees from TACF.s backcross breeding program. Dormant stems were collected from 26 of the most promising individual trees on the Meadowview Research Farms in Meadowview, Virginia and attempted to establish them in aseptic tissue culture. Of the 26 trees, 19 produced fresh green shoots during the shoot-forcing stage. Of those 19 lines, 14 were established in aseptic tissue culture. As of November 1, 2013, 10 lines remain growing in aseptic culture. However, only four lines are multiplying well enough to be used in rooting experiments. So far neither the American chestnut rooting protocol or the Chinese chestnut protocol have worked well for rooting the B3F3 lines, so a new protocol will need to be developed for field-ready trees to be produced.

Principal Investigator(s) and Institutional Affiliation(s)

Allison Oakes
Ph.D. Graduate Student
State University of New York, College of Environmental Science and Forestry, Department of Environmental Forest Biology, 1 Forestry Dr, Syracuse NY 13210

Charles A. Maynard
Professor
State University of New York, College of Environmental Science and Forestry, Department of Forest and Natural Resources Management, 1 Forestry Dr, Syracuse NY 13210

William A. Powell
Professor
State University of New York, College of Environmental Science and Forestry, Department of Environmental Forest Biology, 1 Forestry Dr, Syracuse NY 13210

Current Progress

Fourteen out of the twenty-six individual trees from which cuttings were collected in February 2013 were established in sterile tissue culture. Four lines were lost due to lack of growth in tissue culture. The ten surviving lines are shown in Table 1. Two lines, NV 732 and NX 1385, are multiplying well in American chestnut pre-rooting medium. The other ten lines are alive but growing moderately or very slowly on American chestnut pre-rooting medium (Figures 1 & 2). Two to four shoots from each of the twelve lines have been placed on Chinese chestnut pre-rooting medium to see if growth could be accelerated (study in progress).

The shoot cultures from the Meadowview collection are not growing as vigorously as either American or Chinese chestnut, but they do look very much like other B3F3 cultures taken several years ago from trees at the Lafayette Road Experiment Station.

The rooting process will need to be modified as well, since Chinese chestnuts have an aversion to activated charcoal in rooting media. Our B3F3s have been difficult to root using the American chestnut protocol, and require activated-charcoal-free post rooting medium.

We have had limited success with using a modified American chestnut rooting protocol which eliminates the activated charcoal in the rooting medium. We currently have four lines that could be rooted and are now in the growth chamber for acclimatization. We are still limited by the slow growth in culture of the lines, which reduce the number of shoots available for rooting. By contrast, we generally root 30-50 shoots per line of our American clones as opposed to 2-4 of the B3F3 shoots. There is also a significant loss between the rooting and potting stages where many of the plantlets that were rooted die before finishing the post-rooting treatment and are subsequently not potted (Figure 1.) None of the plantlets are yet large enough to be moved to the greenhouse so are still at risk of not completing acclimatization.

Table 1. Production (rooting and acclimatization) of chestnut B3F3 lines

	Current # in Production (Growth chamber)	Shoots in tissue culture	Total Rooted to date	Total Potted to date	Total Moved to GH to date
NV 77	**2**	12	4	2	0
NV 284	0	8	4	0	0
NV 725	0	3	0	0	0
NV 732	**3**	20	3	3	0
NX 618	0	2	0	0	0
NX 834	0	14	3	0	0
NX 1385	**2**	22	11	4	0
NX 1502	0	2	0	0	0
PS 1708	0	4	0	0	0
PJ 2688	**4**	5	5	4	0

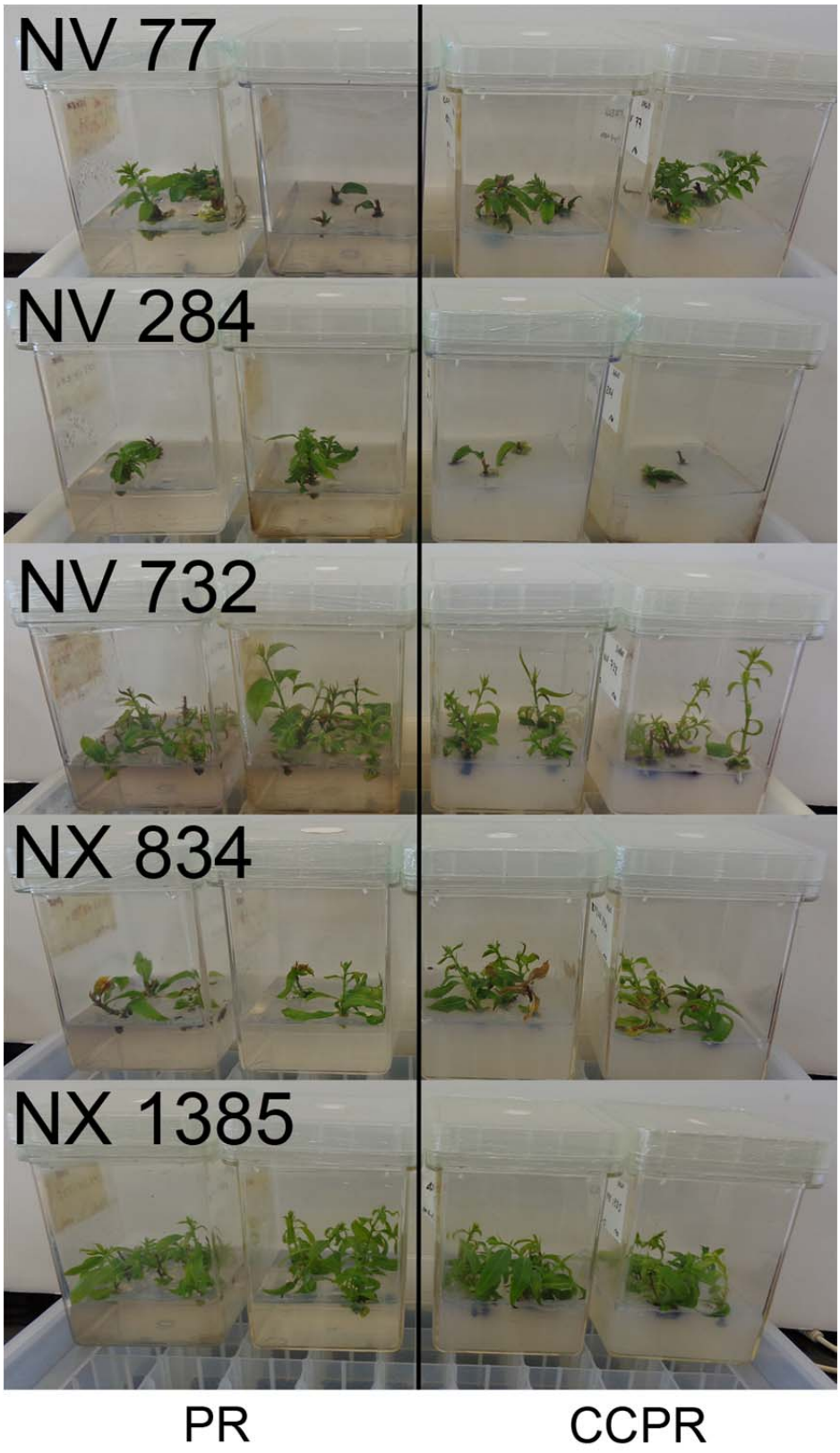


Figure 1. Fast-growing lines in American chestnut pre-rooting medium (PR) and Chinese chestnut pre-rooting medium (CCPR) after three weeks.

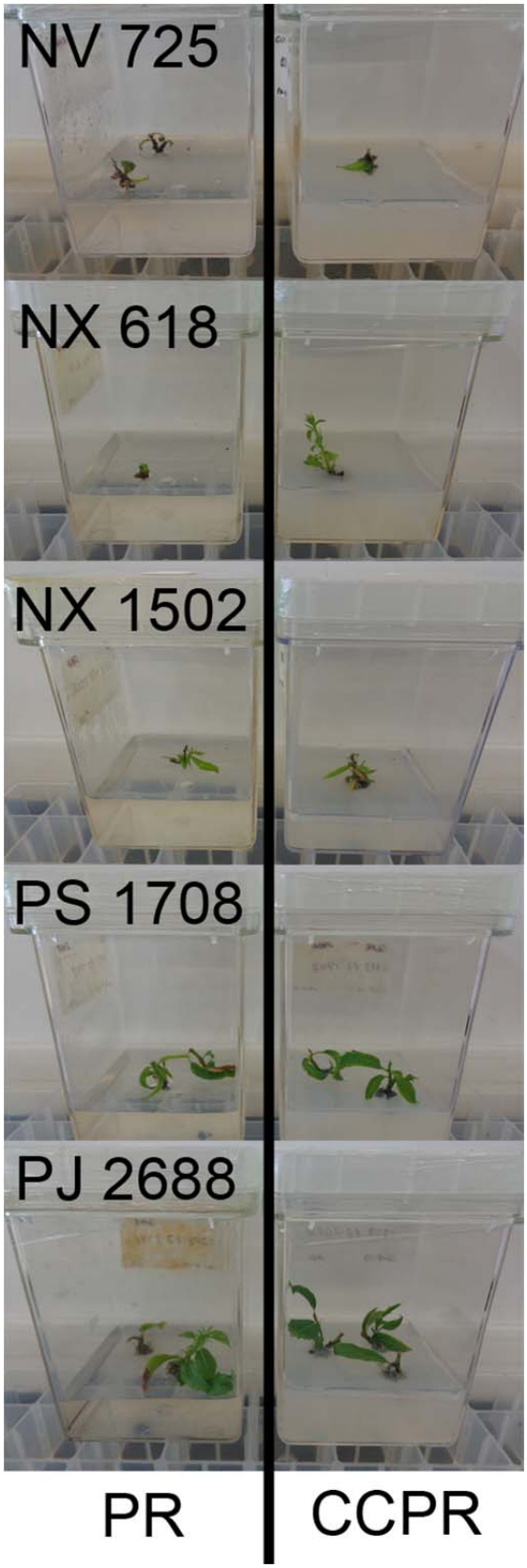


Figure 2. Poorly growing lines in American chestnut pre-rooting medium (PR) and Chinese chestnut pre-rooting medium (CCPR) after 3 weeks.

Future Work:
We will continue multiplying the ten lines in both PR and CCPR. We will soon have enough shoots of the NV 77, NV 732, NX 834 and NX 1385 to begin rooting experiments with enough replicates to detect statistically significant differences among treatments. We intend to compare a "pulse" auxin treatment to our standard "dip" auxin treatment. Instead of dipping the cut end of the shoot in 10mM IBA and immediately placing in a growth-regulator-free rooting medium, the shoots will be placed in an auxin-containing low-salt rooting medium for 24-48 hours and then moved to a growth-regulator-free post-rooting medium. This has worked well in the past for certain varieties of American chestnut (Serres et al. 1990).

We will also continue transferring the slow-growing lines in the two pre-rooting media to see if the lines improve over time. We may lose NV 725 and NX 1502, as they have not produced any fresh growth during the most recent transfer. Failure to grow is how we lost the four lines that were established in culture but are no longer listed.

Breakdown of how funds were spent

$565	Labor, including shoot forcing procedure, media prep, micropropagation
$850	First trip to Meadowview, VA in Nov 2012
$850	Second trip to Meadowview, VA in Feb 2013
$50	Materials for collection
$2265	Total
$450	Remaining for materials. Expect to publish in TACF Journal.

Distribution of results to date

Oakes, A.D., Powell, W.A., Maynard, C.A. *Preservation and multiplication of elite backcross American chestnut hybrids by micropropagation.* Presentation given at the annual New York State American Chestnut Foundation Meeting; 2013, Oct 11-13, Syracuse NY.

Oakes, A.D., Powell, W.A., Maynard, C.A. *Preservation and multiplication of elite backcross American chestnut hybrids by micropropagation.* Poster presented at the annual American Chestnut Foundation Meeting; 2013, Oct 18-20, Herndon VA.

References

Serres, R., P. Read, W. Hackett, and P. Nissen. 1990. “Rooting of American Chestnut Microcuttings.” *Journal of Environmental Horitculture* 8 (2): 86–88.

Medium recipes

American chestnut Pre-Rooting medium (PR)

Label: 'PR-batch#'

Protocol (To make 100ml working solution):

1.	Add approx. 500 ml H_2O to 1500 ml beaker.	
	(Add following with constant stirring):	
2.	WPM salts	2.3 g
3.	Nitsch and Nitsch vitamins	109 mg
4.	MES	1.0 g
5.	PVP-40	1.0 g
6.	BA Stock	220 ul (of a 1.0 mM stock)
7.	Sucrose	30 g
8.	Bring volume to 1000 ml	
9.	Adjust pH to 5.5 $\pm$ .05 with 1.0 N KOH.	
10.	Add Phytagel	3.5 g/l
11.	Microwave until dissolved and mix thoroughly.	
12.	Pour into cubes (85mL) and cap with vented lids.	
13.	Fill out Medium Batch Form and attach sterility indicator tape.	
14.	Autoclave (20 minutes).	
15.	Cool in hood.	

American chestnut Rooting medium (RT)

Label: 'RT-batch#'

Protocol (To make 100ml working solution):

16.	Add approx. 500 ml H_2O to 1500 ml beaker.	
	(Add following with constant stirring):	
17.	MS salts (1/2 strength)	2.15 g
18.	Sucrose	30 g
19.	Bring volume to 1000 ml	
20.	Adjust pH to 5.5 $\pm$ .05 with 1.0 N KOH.	
21.	Add Phytagel	3.5 g/l
22.	Microwave until dissolved and mix thoroughly.	
23.	Fill out Medium Batch Form and attach sterility indicator tape.	
24.	Autoclave (20 minutes).	
25.	Cool in water bath until 55°C.	
26.	Mix in 2g autoclaved activated charcoal.	
27.	Pour in the hood into 6 disposable take out containers (~150mL), close containers.	
28.	Cool in hood.	

American chestnut Post-Rooting medium (POR)

Same as PR, but made in disposable take out containers instead of Magenta vessels.

Chinese chestnut Pre-Rooting medium (CCPR)

Label: 'PR-batch#'

Protocol (To make 100ml working solution):

29. Add approx. 500 ml H_2O to 1500 ml beaker.
    (Add following with constant stirring):
30. WPM salts 2.3 g
31. Nitsch and Nitsch vitamins 109 mg
32. BA Stock 220 ul (of a 1.0 mM stock)
33. IBA Stock 220 ul (of a 1.0 mM stock)
34. Sucrose 30 g
35. Bring volume to 1000 ml
36. Adjust pH to 5.5 $\pm$ .05 with 1.0 N KOH.
37. Add Agar 7 g/l
38. Microwave until dissolved and mix thoroughly.
39. Pour into cubes (85mL) and cap with vented lids.
40. Fill out Medium Batch Form and attach sterility indicator tape.
41. Autoclave (20 minutes).
42. Cool in hood.

Chinese chestnut Rooting medium (CCRT)

Label: 'RT-batch#'

Protocol (To make 100ml working solution):

43. Add approx. 500 ml H_2O to 1500 ml beaker.
    (Add following with constant stirring):
44. MS salts (1/2 strength) 2.15 g
45. Sucrose 30 g
46. Bring volume to 1000 ml
47. Adjust pH to 5.5 $\pm$ .05 with 1.0 N KOH.
48. Add Agar 7 g/l
49. Microwave until dissolved and mix thoroughly.
50. Fill out Medium Batch Form and attach sterility indicator tape.
51. Autoclave (20 minutes).
52. Cool in water bath until 55°C. Do NOT add activated charcoal.
53. Pour in the hood into 6 disposable take out containers (~150mL), close containers.
54. Cool in hood.

Chinese chestnut Post-Rooting medium (CCPOR)

Same as PR, but made in disposable take out containers instead of Magenta vessels.

American chestnut Rooting Protocol (revised 10-28-13, AO)

Equipment and supplies:

1. Small equipment set up: 2 scalpels, and 1 each of the following; (bayonet forceps, needle-point forceps, alcohol dip tube, all sterile)
2. Laminar-flow hood
3. Sterile small Petri dishes
4. 10mL syringe
5. 0.2 µM Filter sterilizer tip
6. Sterile IBA dipper, in Al-foil wrapped glass petri dish
7. **10mM** IBA (not filter sterilized)
8. Cubes of American chestnut plantlets in Pre-Rooting (PR) Medium
9. Sterile cubes of PR medium
10. Sterile distilled H_2O
11. Sterile disposable take out containers of Rooting medium (RT) ("clamshells" or "clams")

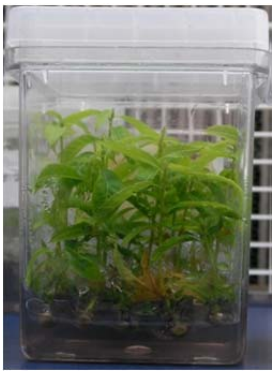
American Chestnut Rooting Protocol

Part 1. Culture maintenance and rooting prep

1. Starting with healthy and vigorously growing chestnut shoot cultures in PR.

2. Transfer 1-2cm shoots to Magenta GA-7 vessels with vented lids containing PR medium (Figure 1), maximum 9 shoots per cube. Incubate shoots on a 16-hour photoperiod for 3-4 weeks.

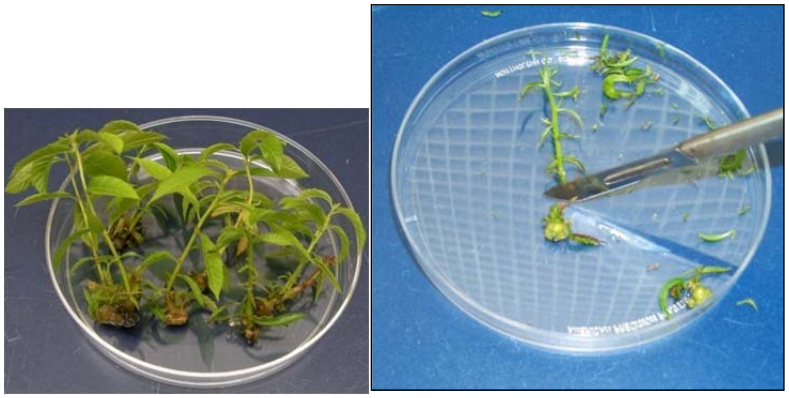
3. Wait for a majority of the shoots are at least 3 cm in length and have more than 4 large leaves (3-4 weeks).The more leaves a shoot has, the more likely it is that it will survive acclimatization. *Note*: This may take two or three transfers. The purpose of this pre-rooting treatment is to promote the elongation of single shoots rather than multiple-shoot clumps.



Part 2. Rooting of chestnut microshoots

1. Prepare your IBA dip. Using sterile forceps, place the dipping rack into a small sterile Petri dish. Use the syringe to suck up 10mL of 10mM IBA. Affix the filter tip onto the syringe and push the filtered solution through it into the petri dish with the dipping rack inside.

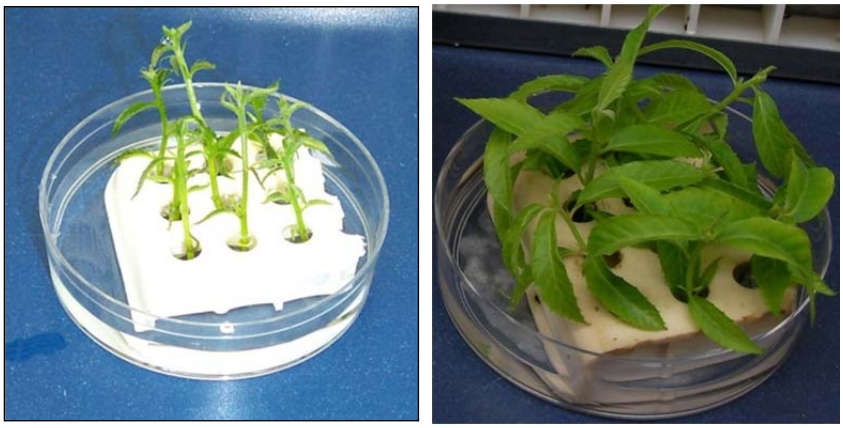
2. Select PR cubes that contain tall, healthy shoots. Place shoots that are 2 cm or longer with lots of large healthy leaves in a sterile Petri dish or a sterile paper towel. Remove the leaves on the lowest 1cm of stem and cut the callus off the base.



Removal of callus.

3. With a sharp scalpel, carefully divide the basal end of the shoot 1 to 2 mm up the stem. Make sure you are slicing through the stem and not crushing the tissue.
Alternatively, cut a 45° angled end on the base of the tip to increase vascular surface area. Try to avoid exposing the cut tip to air to decrease embolisms forming in the vascular tissue.

4. Immediately place shoots into the rack in the IBA-filled Petri dish, making sure that all shoots are submerged at their base. Start the timer for 1 minute. If you observe any leaf wilt, move the plantlets into the rooting media immediately and shorten the time in IBA. *Note*: Avoid getting IBA high on the shoots or leaves, as this can cause excessive callus growth.



5. Immediately after the 1-minute dipping treatment, move each shoot to charcoal Rooting Medium (10-12 shoots per clam). Culture for 4 days in complete darkness at room temperature (a dark cabinet).

6. Extra stem tissue and small shoots (<2cm) left over from can be placed into a fresh cube of PR. These will be rooted during the next cycle.

7. After 4 (3 minimum, 5 maximum if needed) days, remove shoots from Rooting (RT) Medium and transfer them to Post-Rooting (POR) Medium. Roots may not be visible - more roots will be produced during this culture cycle.

8. Culture in POR for 10-14 days before potting.

Chinese Chestnut Rooting Protocol

Same as American, but use CCPR in place of PR, CCRT in place of RT, and CCPOR in place of POR.

Final Report: Preservation and multiplication of elite backcross American chestnut hybrids by micropropagation

Summary

One of the bottlenecks for the breeding program that is dealing with quantitative resistance is the ability to quickly multiply the B3F3 trees demonstrating the highest levels of blight resistance. This study collected dormant stems from the twenty-six of the most promising individuals from the TACF breeding program and attempted to clonally establish them in aseptic tissue culture. Of the cuttings taken from 26 trees, 19 produced fresh green shoots during the shoot-forcing stage. Of those 19 lines, 14 were established in aseptic tissue culture. Field-ready young trees will be available for field plantings throughout the country in the spring of 2014 and can be incorporated into any of the state chapter's breeding programs.

Principal Investigator(s) and Institutional Affiliation(s)

Allison Oakes
Ph.D. Graduate Student
State University of New York, College of Environmental Science and Forestry, Department of Environmental Forest Biology, 1 Forestry Dr, Syracuse NY 13210

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Professor
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William A. Powell
Professor
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Current Progress

Fourteen out of the twenty-six individual trees from which cuttings were collected in February 2013 have been established in sterile tissue culture. The exact tree lines are shown in Figure 1. These cultures will be multiplied and rooted over the summer, a limited number of acclimated plantlets may be available for distribution for the fall planting in 2013 and in larger numbers for spring planting in 2014.

The first attempt at taking cuttings in November 2012 was unsuccessful due to multiple factors. The main problem was that November in southern Virginia is not far enough into winter to reach cooler temperatures needed to reduce the contamination load. By February cold temperatures had

killed off more potential contaminants. Also, the cuttings taken in February developed fresh green shoots a week faster than those taken in November, since the cuttings were closer to flushing. This meant that any contaminants that survived the initial bleach soak had less time to become a problem while the cuttings were being forced.

Lessons learned from the first trial were critical to the success of the second. After taking the second round of cuttings, immediately upon arriving at Syracuse all cuttings were washed in a detergent solution and then placed in a 20% bleach soak for ten minutes before being rinsed and placed in the shoot forcing jars. This immediate sterilization dramatically reduced contamination of the shoot forcing solution and fungal growth on the fresh shoots, which was a major complication in the first trial.

Also, the forced shoots were put into sterile culture in groups of six, rather than all thirty at once. Working at a slower pace with more attention to the individual cultures resulted in a much lower contamination rate. Nineteen of the tree lines produced fresh, green shoot tissue from the stem buds after four weeks in the lab. Out of the 19 lines that did produce fresh green shoots, 14 of them were successfully established in shoot culture for a 73% success rate. Only the fresh green tissues (stems, shoot tips, leaves) were put into sterilization, no bark tissue or leaf scales were sterilized along with the shoots. This may have helped keep the tissue clean. The remaining seven tree lines had not formed shoots by 3/27/13, so buds were excised from stems, sterilized, and place on tissue culture medium. Unfortunately, using the excised buds was not successful, even with stringent sterilization the contamination overwhelmed the buds in culture. The inclusion of bark may have contributed to the contamination load.

Tree lines that were not successfully put in culture were bleach rinsed again and kept in shoot forcing stage to see if another flush of fresh tissue will emerge, however a grey mold took over the cuttings within the following two weeks. In conclusion, the window for shoot forced cuttings is three to four weeks, and it is unlikely that cuttings will flush after that time without being contaminated.

Tree	Fresh Shoots? (3/27)	Put in culture (3/27)	Status (4/1)	Rescued (4/1)	Successful Rescue (4/5)	Status (4/5)	Rescued (4/5)	Successful Rescue (4/18)	Status (4/18)
NV 77	y	x	clean			clean			in culture
NV 156	y	x	clean			dead			
NV 189	y	x	con*	n		dead			
NV 282	y	x	con	n		dead			
NV 284	y	x	clean			clean			in culture
NV 520	n	x	con	n		dead			
NV 725	y	x	con	y	y	clean			in culture
NV 732	y	x	clean			clean			in culture
NX 117	y	x	con	y	n	con	y	y	in culture
NX 290	n	x	con	n		dead			
NX 618	y	x	clean			clean			in culture
NX 620	y	x	clean			con	y	y	in culture
NX 834	y	x	con	y	y	clean			in culture
NX 854	n	x	con	n		dead			
NX 857	y	x	con	y	n	con	y	y	in culture
NX 1293	y	x	con	n		dead			
NX 1351	y	x	con	n		dead			
NX 1385	y	x	clean			clean			in culture
NX 1411	n	x	con	n		dead			
NX 1413	n	x	con	n		dead			
NX 1502	y	x	con	y	y	clean			in culture
PS 1708	y	x	clean			clean			in culture
PJ 2688	y	x	clean			clean			in culture
PJ 3663	n	x	con	n		dead			
PJ 4182	y	x	con	y	y	clean			in culture
PS 5347	n	x	con	n		dead			

Figure 1. Status of the B3F3 tree lines. * denotes contaminated with fungi or bacteria. Some lines were too contaminated to rescue on 4-1-13.

Future Work

The shoots which are in culture are not growing in a similar fashion to either American or Chinese chestnuts; they look very much like other B3F3 cultures taken from trees at the Lafayette Road Experiment Station. Once enough shoots are available we will try placing them in Chinese chestnut multiplication and pre-rooting medium. In addition, it would be advisable to try a small media factorial to see whether modifying the auxin-cytokinin ratio would promote better multiplication and elongation for the hybrid cultures.

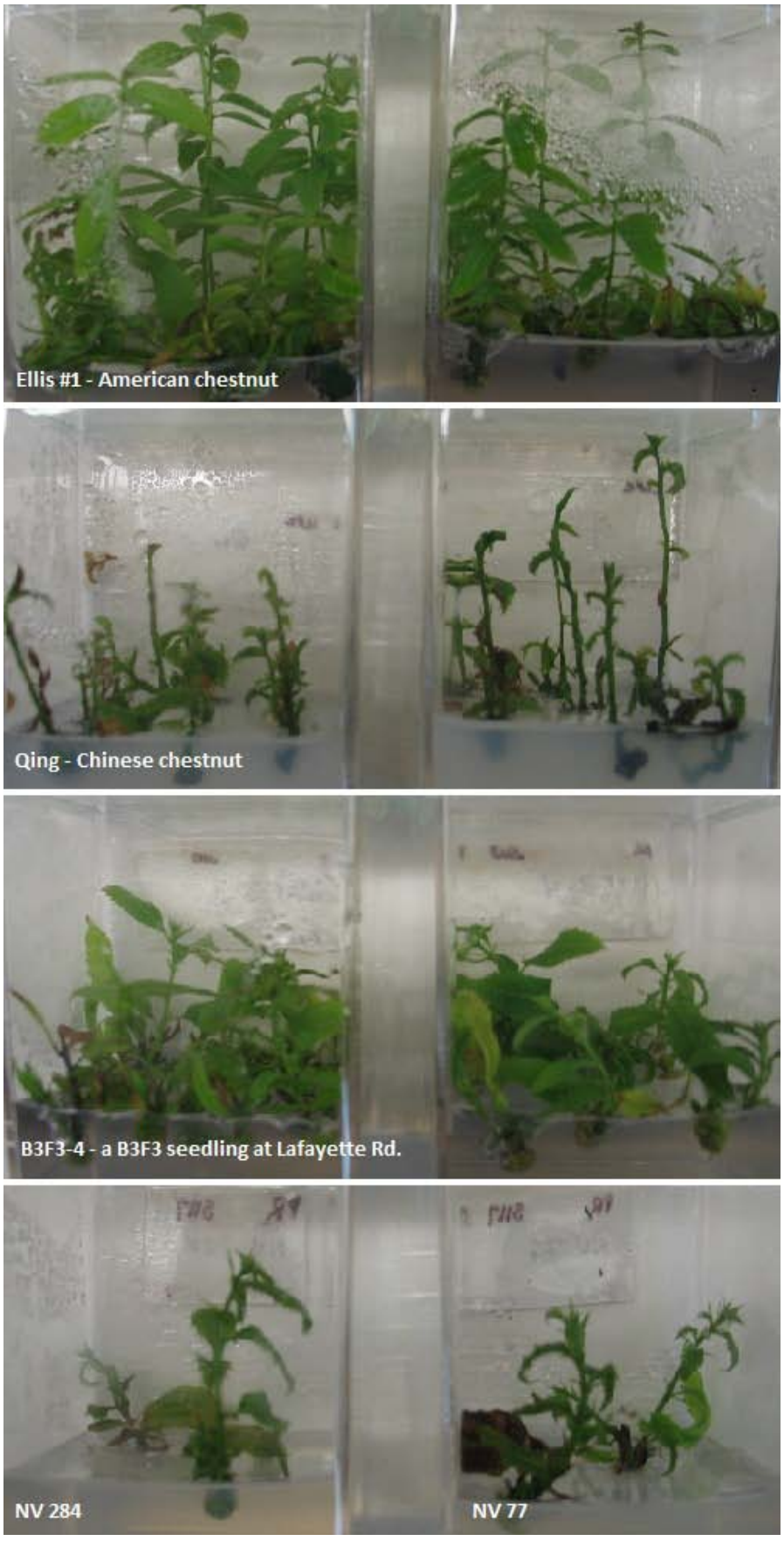


Figure 2. American, Chinese, and hybrid chestnuts in tissue culture. The American and hybrid chestnuts are in PR (American-prerooting) medium, the Chinese chestnuts are in CCPR (Chinese prerooting) medium.

The rooting process will need to be modified as well, since Chinese chestnuts have an aversion to activated charcoal in rooting media. Our B3F3s have been difficult to root using the American chestnut protocol, and require activated-charcoal-free post rooting medium.

Recommendations for Future Shoot Forcing Projects

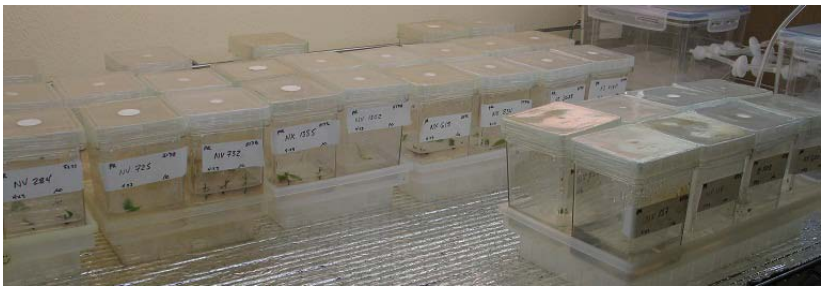
The two protocols for cleaning the cuttings and for putting the forced shoots in culture are attached. Our main recommendation would be to immediately sterilize the cuttings on site with the detergent, bleach rinse, and drying overnight in a hood. Then the shoots could be easily shipped.

Breakdown of how funds were spent

$565	Labor, including shoot forcing procedure, media prep, micropropagation
$850	First trip to Meadowview, VA in Nov 2012
$850	Second trip to Meadowview, VA in Feb 2013
$50	Materials for collection
$2265	Total
$450	Remaining for materials. Expect to publish in TACF Journal.

Figure 3. Freshly propagated NV 732.

Figure 4. The B3F3 collection on the light bench.



Shoot Forcing Stem Cleaning Protocol
Revised 2/27/13 by Allison Oakes

Materials:

Bunches of 6-12 woody cuttings, secured with a rubber band, labeled with tape and permanent marker.

Clean 100ml Pyrex jars, gallon size plastic baggies, one tub of Tween 20 (10 drops/2L), one tub of 20% bleach, pruners, 70% ethanol spray bottle, rubber bands.

1. Only work with 2-3 bunches of cuttings at a time. Wear gloves.
2. Submerge the bunches of cuttings completely in the detergent solution. Gently wash for 5 min to remove grime and particulate matter from the cuttings.
3. Rinse thoroughly under running tap water.
4. Submerge cuttings in 20% bleach for 10min.
5. Rinse thoroughly under running tap water.
6. Trim the ends of the cuttings at a 45° angle with clippers (sprayed with 70% ethanol between uses)
7. Place cuttings into a clean 100mL Pyrex jar, fill jar with 50mL distilled water.
8. Place in hood (not running) overnight to air dry stems.
9. Pour out discolored water and replace with fresh distilled water.
10. Place the large plastic bag over the cuttings and jar, secure with rubber band.
11. Place bagged jar on light bench. Change water once a week, trim ends once every two weeks. If water becomes discolored change more often.
12. Alternatively, multiple bunches of cuttings can be placed together in a larger container with a larger plastic bag for easier water changes.

Shoot-Forced Chestnut Sterilization Procedure
Revised 3/27/13 by Allison Oakes

Materials:
Clippers
250ml Pyrex jars (orange cap)
Captan 50 rinse bucket (2.36g/L)
Tween 20
Bleach solution (20% bleach, 10 drops Tween per L)
Sterile distilled water
Magenta vessels of PR (pre-rooting) medium (see below).

1. Use a scalpel to cut off only the green, freshly growing shoots which have visible stems. Do not collect freshly broken buds, allow them to expand. Place the shoots into a 100ml Pyrex jar.
2. Spray into the jar liberally with 70% ethanol. Swirl to coat well for 30 seconds. Pour off excess.
3. Add 50mL bleach solution. Place on shaker (200RPM) for 15 minutes.
4. From this point on, work in the hood aseptically!
5. In the hood, spray down the jar with 70% ethanol and pour off the bleach solution into a labeled waste container.
6. Pour in 50mL sterile distilled water. Cap tightly! Place on shaker (200RPM) for 5 min. Spray down jar when returning it to the hood, and pour off water into the waste container in the hood.
7. Repeat the distilled water rinse for a total of three rinses. Make a mark on the label for each rinse.
8. Using sterile forceps, remove the shoots from the jar and place on sterile paper napkin.
9. Cut off any large leaves, and cut into 2-3 bud segments.
10. Place no more than four segments per cube of PR media, leaving lots of space between pieces.
11. Wrap cube with clingwrap, place on light bench. Check daily for contamination.

Pre-Rooting Medium (PR):
Magenta GA-7 vessels with vented lids
Woody Plant Media salts (2.3 g/l)
Nitsch & Nitsch vitamins (109 mg/l)
MES (0.5 g/l)
PVP-40 (0.5 g/l)
BA (220 uL of 1 mM - 0.22 uM final)
Sucrose (30 g/l)
pH to 5.5 with 1M KOH
Phytagel (3.5 g/l)