Chestnut Chat: Cloning and Embryogenesis 101

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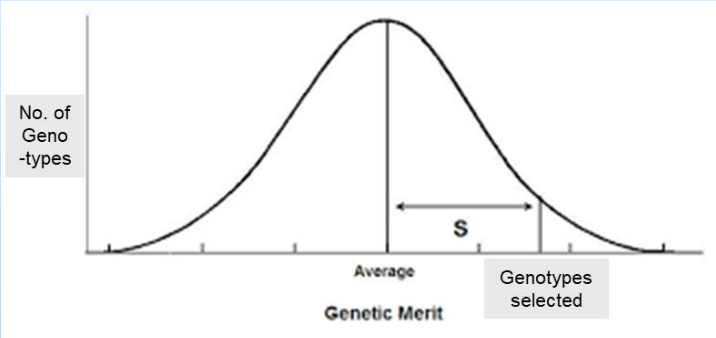
Breeding/production strategies/directions for TACF

- Half-sib technology (current TACF approach)
- Full-sib technology—controlled mass pollination (CMP)—adaptable for chestnut?
- Clones (“varietals”)
  - Rooted cuttings (macropropagation)
  - *In vitro* propagation (micropropagation-broad sense)

Varietal (or clonal) technology +s and –s compared to conventional seedlings

Advantages

- Captures all components of genetic variance (i.e. all superior genes)
  - "Clonal repeatability" is a powerful estimator of heritability of desired traits
- Can apply very high selection differential (choose the "best of the best" genotypes to propagate)



Disadvantages

- High labor and operating expense
- Requires specialized equipment and facilities
- Propagules need to be containerized (no direct sowing)
- Success depends on species, age of starting material and other factors

Rooted cuttings for forest tree propagation

Radiata pine in New Zealand

Photo courtesy of Dr. Dale Smith

Radiata pine “stool-bed” for rooted cutting production

Photo courtesy of Dr. Dale Smith

Rooting beds for Radiata pine cuttings

Photo courtesy of Dr. Dale Smith

Ease of rooting depends on species, genotype, source tree age and other factors— Chestnut is NOT radiata pine




Chestnut rooted cuttings from B3F3 stump sprouts

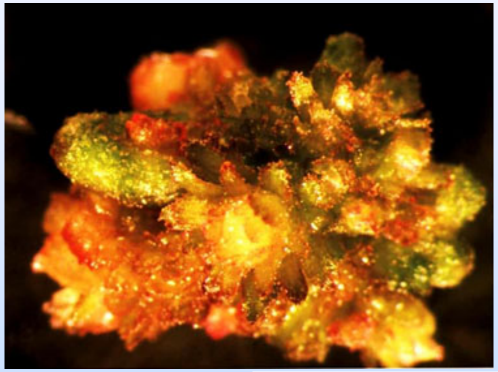
Overall success rate: 0.6%--currently NOT a mass propagation system

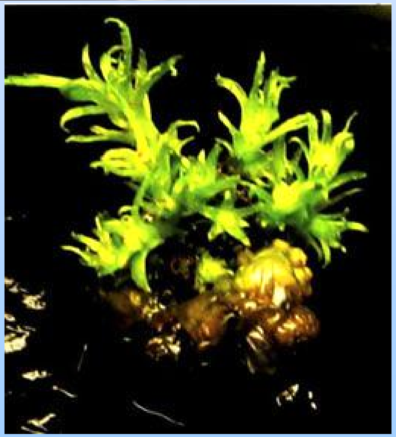
In Vitro Propagation Approaches

- Micropropagation (Axillary shoot culture)
- Organogenesis (Adventitious shoots)
- Somatic embryogenesis

Micropropagation
Like rooted cuttings—some species are easier to micropropagate than others

Eastern cottonwood—very easy to micropropagate.

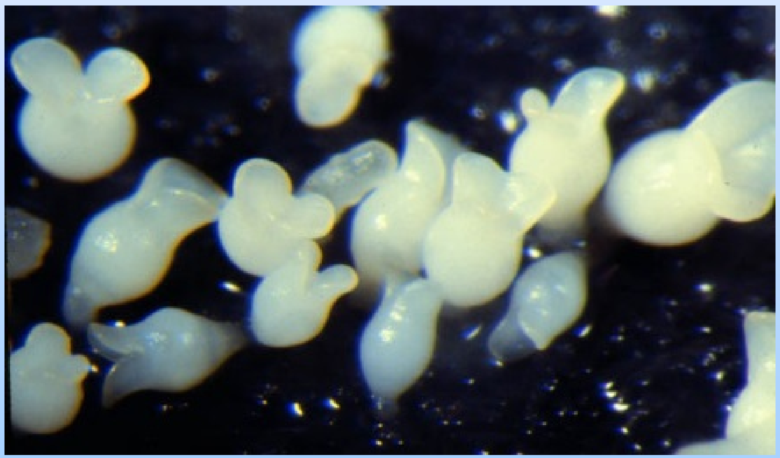




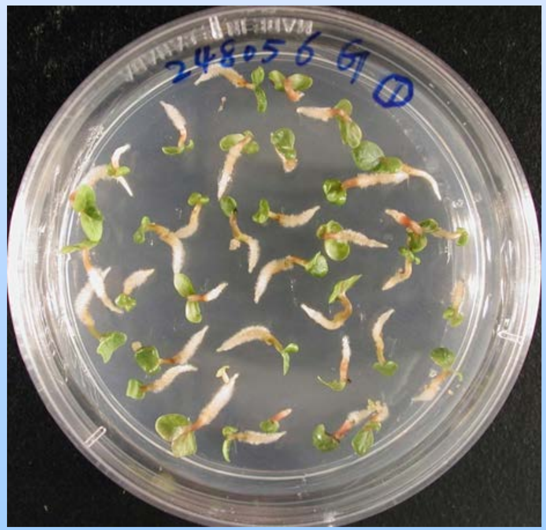
American chestnut micropropagation—doable, but not so easy—<u>especially rooting step</u>, highly genotype-dependent and almost no reports for mature trees.

Somatic Embryogenesis (SE):

A process by which structures (“somatic embryos”) resembling seed embryos are produced asexually. These somatic embryos can be germinated like seeds to produce clonal seedling-like plantlets (“somatic seedlings”)



Yellow-poplar somatic embryos



Germinating somatic embryos

Somatic seedlings

Somatic embryogenesis +s and -s

Advantages over other cloning approaches:

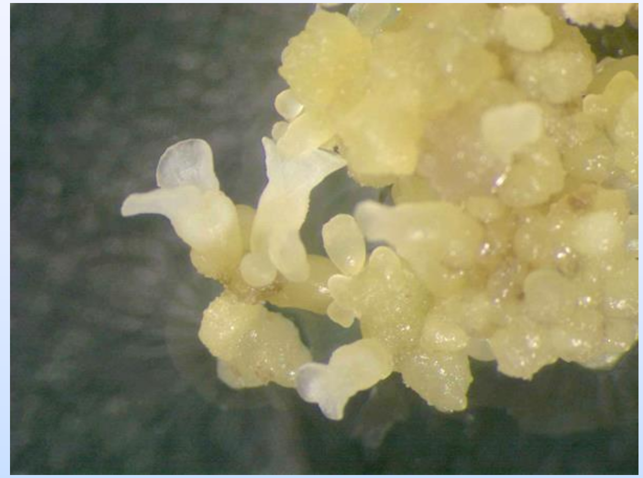
- Powerful combination with full-sib breeding
- Massive potential multiplying power--potential for scale-up and automation
- Propagules have taproots, like true seedlings
- Ability to hold germplasm indefinitely using cryostorage
- Can be used as target material for gene transfer

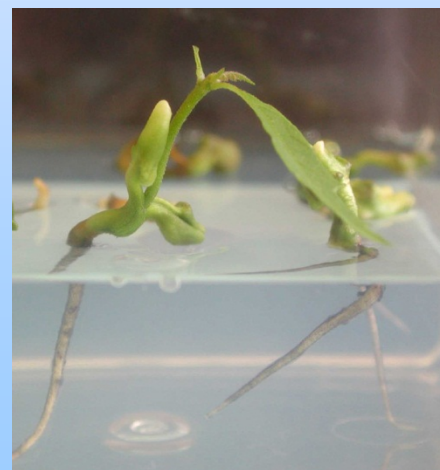
Disadvantages compared to other clonal propagation approaches:

- Major bottlenecks remain in chestnut SE process
- Cultures cannot currently be started from mature tree tissues, so cannot directly clone proven genotypes

Development of American chestnut SE technology was started at UGA in 1989

- 1989 – first embryogenic cultures established
- 1997 – first somatic seedlings germinated
- 2001 – first somatic seedlings planted
- 2005 – Suspension culture-based system established





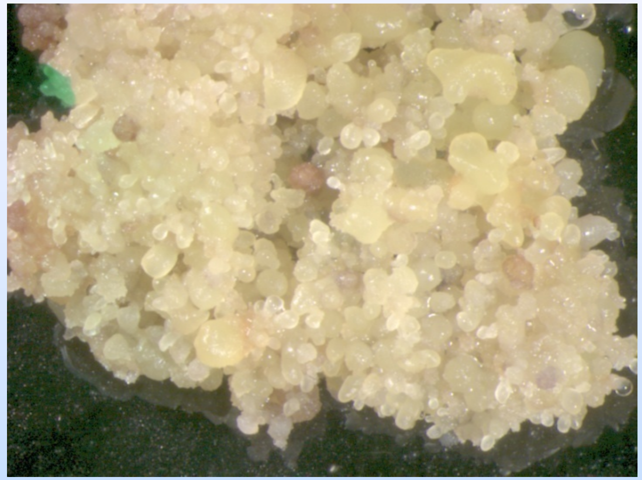
SE protocol was developed for pure American chestnuts, allowing us to conserve and propagate germplasm from Large Surviving American (LSA) chestnuts

Thompson and Ragged Mountain Trees

Amherst Tree

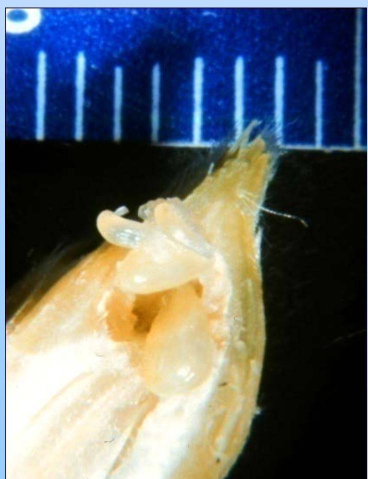
Adair County Tree

Cultures are initiated from immature seeds--only an average 2% success rate, but can get at least some embryogenic cultures from almost any American chestnut genotype

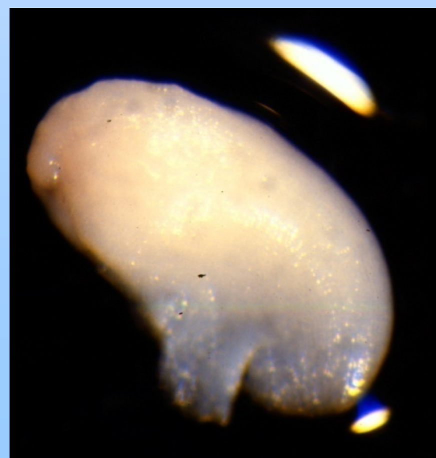


January – “Capture”

November



August -- Culture Initiation

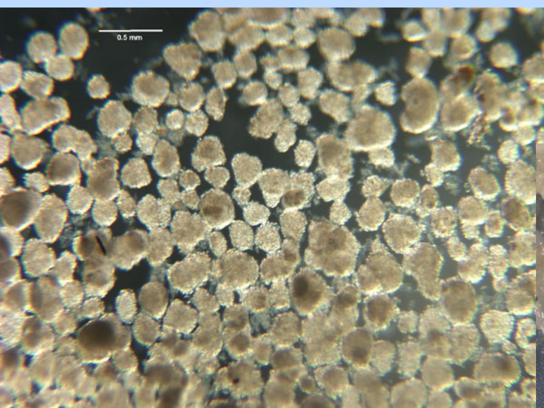


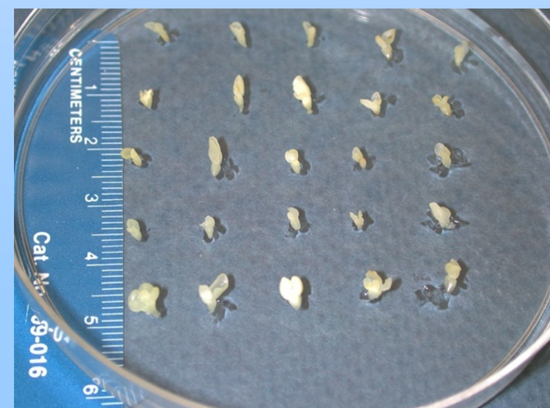
October

American Chestnut Medium Recipe

- Lloyd and McCown’s Woody Plant Medium major salts (N, P, K, Ca, Mg)
- Lloyd and McCown’s Wood Plant Medium minor salts (Mn, Zn, Cu, B, Mo)
- Murashige and Skoog’s Iron (Fe)
- Schenk and Hildebrandt’s vitamins (thiamine, nicotinic acid, pyridoxine)
- 30 g/l sucrose
- 1 g/l casein hydrolysate <u>or</u> 0.5 g/l L-glutamine
- 2 or 4 mg/l 2,4-D (auxin)
- Gelled with Phytagel gellan gum

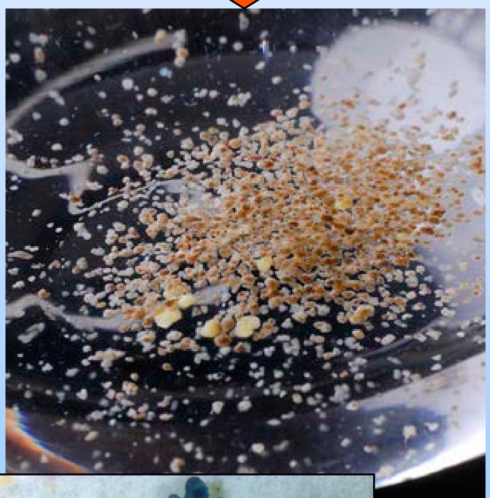
Potential for scale-up using suspension cultures



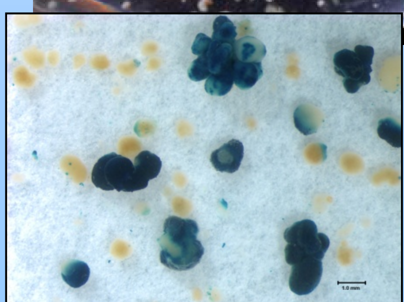


Embryogenic cultures are the route (target material) for gene transfer and gene editing

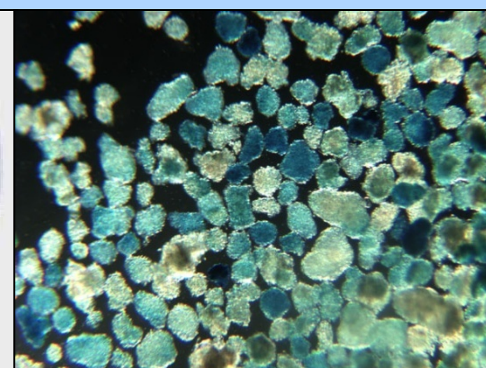
1. Agrobacterium (AGL1) infection of chestnut SE culture



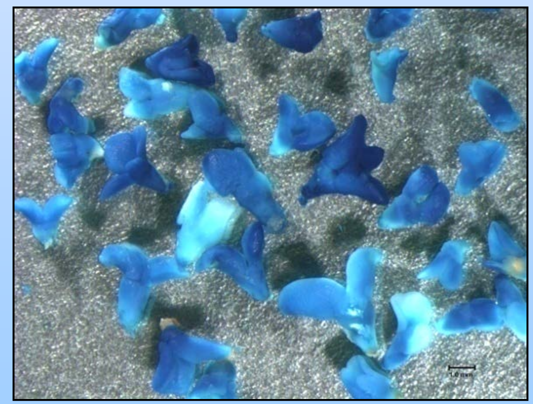
2. Liquid selection with Geneticin prevents “escapes”



3. Proliferation of individual transclones

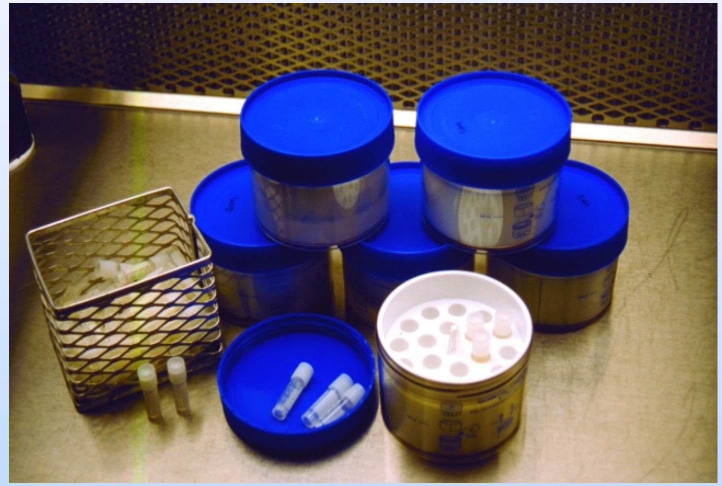


4. Somatic embryo production



5. Somatic seedling production

Once an embryogenic culture is established, it can be stored indefinitely in liquid nitrogen….
…and recovered



1 week 3 weeks Embryo production

2010 TACF Germplasm Agreement with UGA and FHI allowed culturing of TACF BC3F3 material for the first time

THE AMERICAN CHESTNUT FOUNDATION®
GERMPLASM AGREEMENT
Regional Adaptability Breeding Program

This Agreement, dated and effective____________________, 20___, is between The American Chestnut Foundation, a Virginia nonprofit corporation with its principal facility in the Commonwealth of Virginia (hereinafter referred to as "TACF"), and the entity executing this Agreement at the foot hereof (hereinafter referred to as the "Recipient").In addition, this Agreement falls under the context of the attached Memorandum of Understanding between TACF and the Forest Health Initiative (FHI).

The Reasons for this Agreement: TACF is in the process of breeding hybrid chestnut trees for eventual release into the public domain closely resembling pure American chestnut trees but without susceptibility to the disease known as chestnut blight and with resistance to insect pests and other major pathogens of chestnut. The method of plant breeding being used by TACF is commonly referred to as the "backcross method" wherein lines of American chestnut stock are outcrossed once to other species of chestnut carrying genetic resistance to chestnut blight, and successive generations of such outcrosses are then repeatedly backcrossed to American chestnut to recover the desirable characteristics fo the American chestnut tree while incorporating blight resistance. It is in the interests of TACF and of the Recipient to be able to test and observe the characteristics of hybrids which are in the earlier stages of such backcrossing (i.e., the original outcross and first through third backcrosses (and intercrosses between individual tres of the same generation of backcrossing) since selected offspring of third backcross trees are considered to be genetically primarily an American chestnut type of tree). But Recipient and TACF do not want the Recipient or others to use genetic material from such early stages for propagation purposes because: (1) the Recipient and TACF wish to preserve TACF's rights to such genetic material; and (2) the Recipient and TACF *most emphatically* do not want any person to take such material and market it, or to market any progeny from it; the material may not have the characteristics desired or have characteristics that are not consistent with the goal of TACF, namely "the Restoration of the American Chestnut" and not a Chinese or other type of tree; and (3) the Recipient and TACF do not want to be identified with the distribution, increase or marketing of material that has the potential of diluting the resident American chestnut population in the Appalachian mountains.

The Terms of this Agreement: This Agreement applies to all varieties of chestnut germplasm, and includes but is not limited to: pollen, nuts, scion wood, sprouted seeds, small chestnut plants, rooted cuttings, and all progeny thereof, all of which are owned by TACF and hereinafter referred to as the "germplasm".

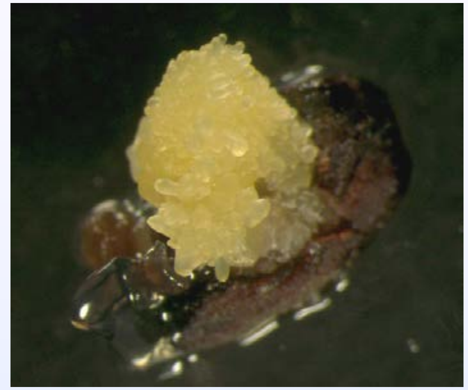
TACF agrees to supply samples of germplasm to the Recipient. In consideration of this action by TACF, the Recipient agrees to abide by the following terms and conditions as to said germplasm and any other germplasm which has heretofore been received or will hereinafter be received from TACF which is not otherwise covered by a subsequent agreement, UNLESS AND UNTIL TACF SPECIFICALLY RELEASES ANY CONDITION IMPOSED BY THIS AGREEMENT ON THE CUSTODY AND USE OF ANY OF SAID GERMPLASM. This agreement supersedes any and all previously signed germplasm agreements between TACF and this recipient.

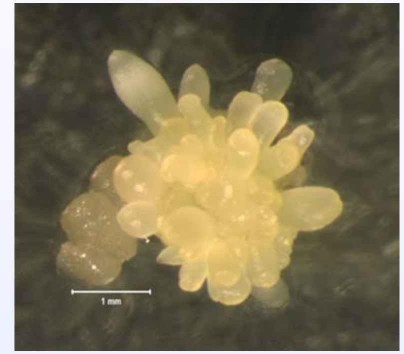
1. The Recipient understands and agrees that this Agreement conveys only a right to carry out research, evaluations and /or field testing on the germplasm on behalf of and in consultation with TACF. None of the germplasm (or any material resulting in any manner from the germplasm) may be sold, offered for sale, given (by gift or otherwise), or in any other manner transferred or distributed to any third party (that is, someone who has not signed a TACF Germplasm Agreement) whatsoever (except as provided in paragraph 7 below) without first being covered by a specific written consent from TACF describing the material sold or otherwise transferred, the conditions of the transfer, and other conditions acceptable to TAACF in its sole discretion. TACF reserves the right to refuse transfer for any reason whatsoever. It is expressly understood that under this Agreement no implied or express license is granted by TACF to the Recipient for any transfer of the germplasm to a third party.
2. The sample of germplasm provided hereunder may be used for basic research, evaluation and/or field testing of behalf of TACF. In vitro tissue cultures may be established using somatic embryogenesis techniques and cultures derived from the material may be employed in genetic transformation experiments. Plantlets produced from the <u>tissue</u> cultures <u>may</u> be grown on UGA property <u>under this germplasm agreement</u> or shared with other Forest Health Initiative cooperators for <u>research</u> purposes if these cooperators <u>first</u> execute their own germplasm agreements with TACF. Selection may be conducted with the germplasm when done as part of a cooperative agreement (or "Selection Agreement") between TACF and the Recipient, with title and distribution right to such selections being retained by TACF.
3. Seed stock increases for evaluation are permitted. However no seed, plants, plant parts seed parts, callus tissue or DNA of or resulting from the germplasm may be transferred or distributed to any third party, except as otherwise provided herein.
4. The Recipient understands that the germplasm is being supplied to the Recipient solely to enable the Recipient to assist TACF in evaluating the germplasm and in furthering the breeding program of TACF. The Recipient agrees to take reasonable care of the germplasm, o make a commitment to the maintenance of the germplasm appropriate to the purposes for which the germplasm has been supplied (and insofar as the Recipient is reasonably able to do so), to cooperate with the State TACF Chapter and TACF so that they may carry out their responsibilities regarding the Regional Adaptability Breeding Program,

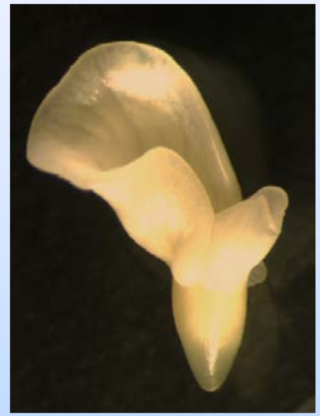
SE protocols developed for American chestnut work OK with TACF hybrid backcross material

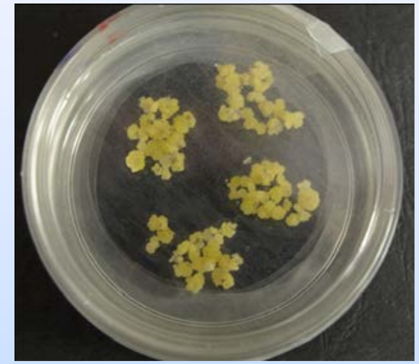
- Embryogenic cultures “captured” for **19 of 20** OP TACF BC3F3 families cultured
- No statistical difference in initiation rates between OP BC3F3 and pure American chestnut
- First CP BC3F3 cultures initiated in August 2012
- Embryogenic cultures captured for **8 of 9** CP TACF BC3F3 families, although initiation rates lower than for OP seeds

BUT…

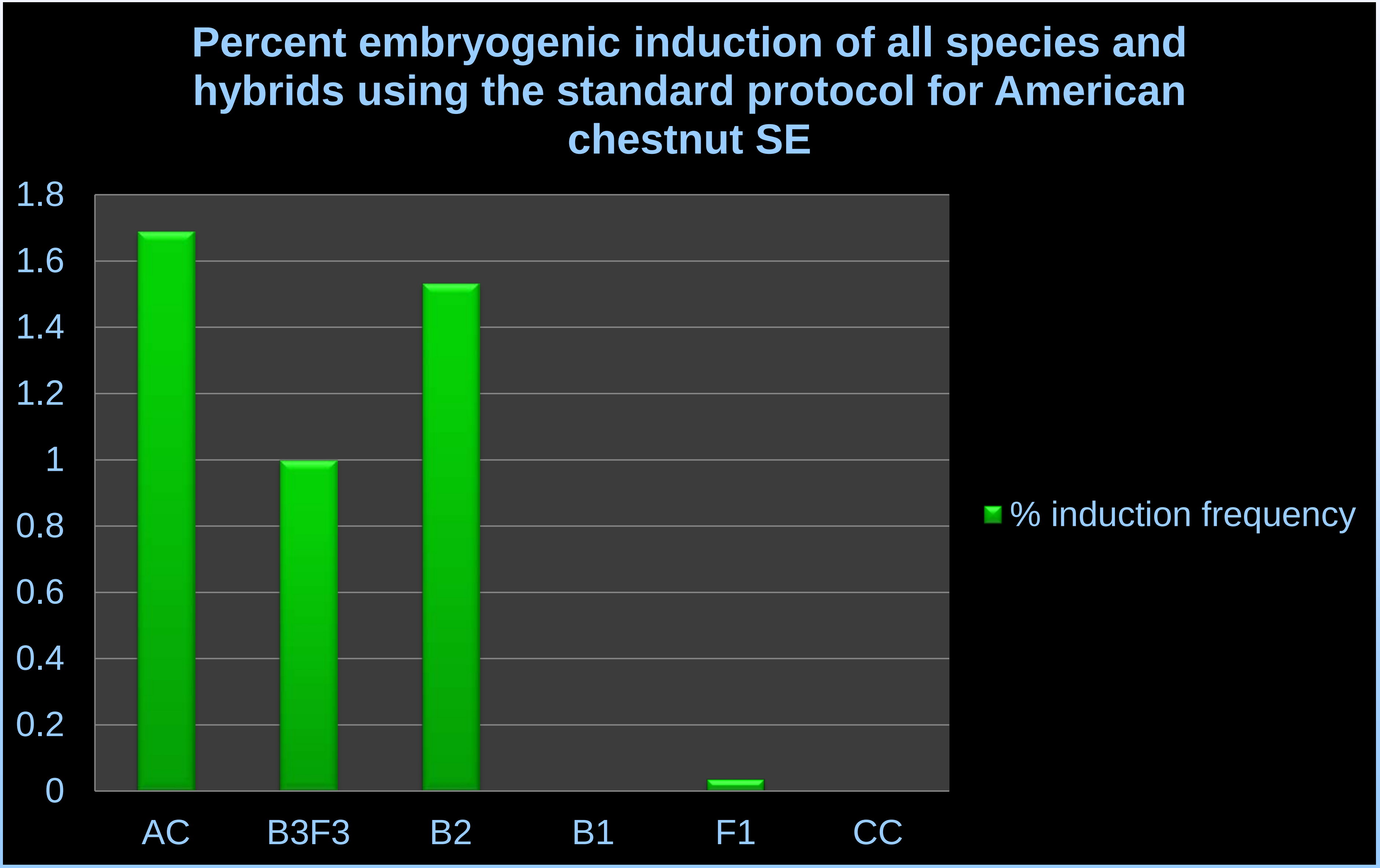




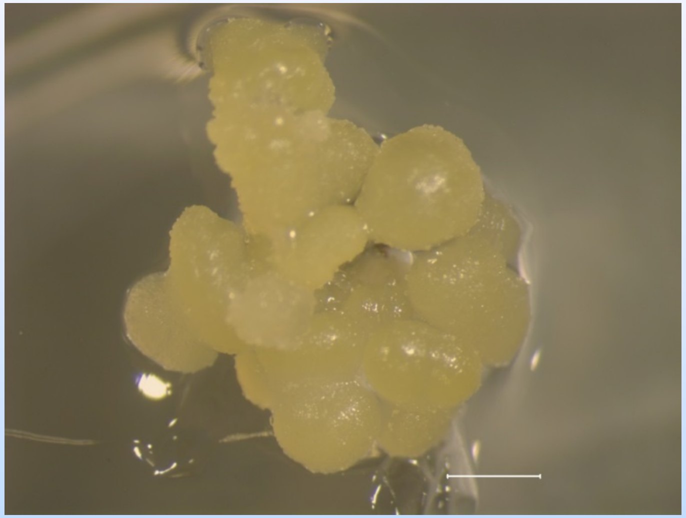


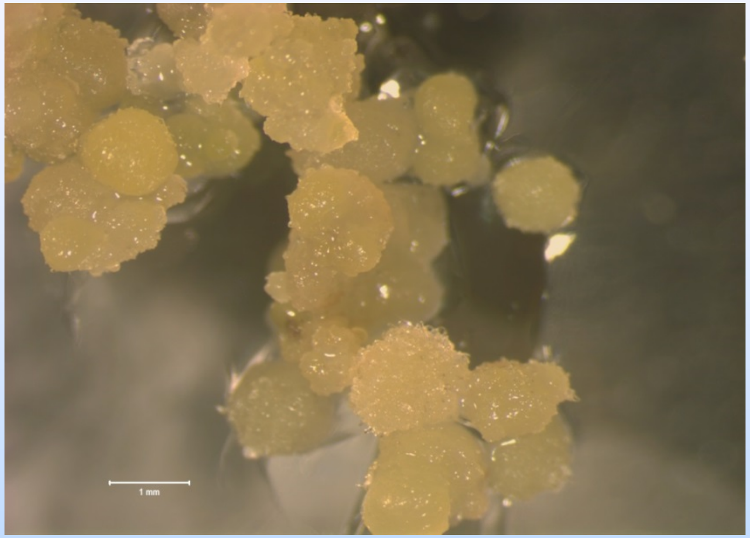


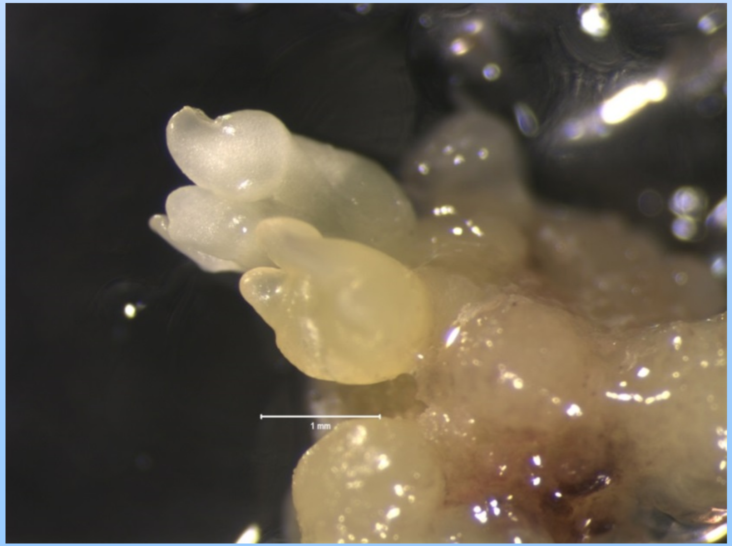
…maybe not so well with genotypes with ≥25% Chinese chestnut genome

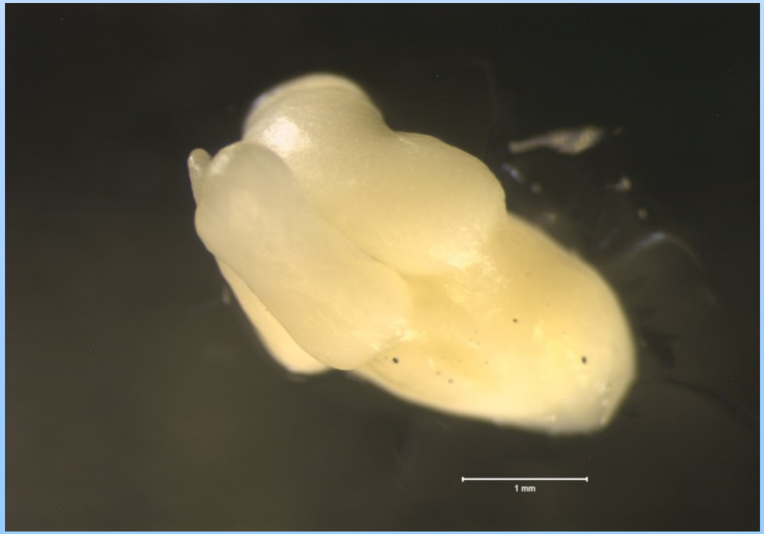


Chinese chestnut embryogenic cultures could be started using a protocol reported for European chestnut SE that uses weaker auxin than 2,4-D



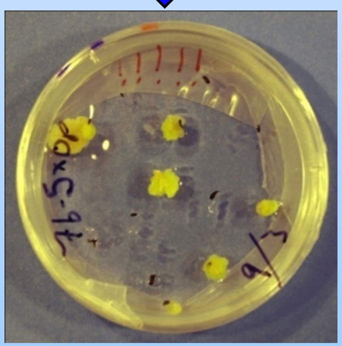




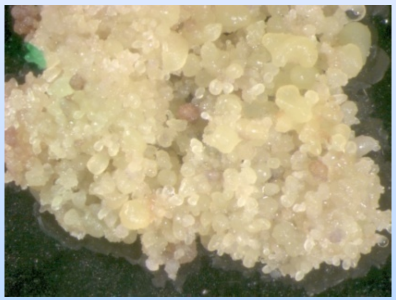


Selection/breeding, SE and cryostorage are a powerful combination for chestnut restoration

Selected B3F2 trees crossed to get B3F3 seeds



B3F3 seeds used to start SE cultures



"Captured" SE cultures

Somatic seedling production

Somatic seedlings screened/field tested

Screening

Results

Copies of all cultures cryostored

Best clones recovered from cryo and mass propagated for planting

Bottlenecks and hurdles to using SE for chestnut propagation

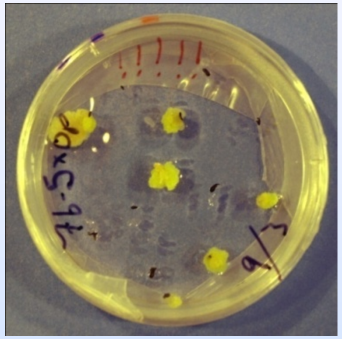
- Long times from culture initiation to somatic seedling production
- Poor somatic embryo quality → incomplete germination
- Somatic seedling losses during acclimatization
- Poor plantlet growth in greenhouse/field—slow root system expansion, short internodes
- Inability to directly clone mature (proven) trees

Timeline from breeding to planted somatic seedlings

2.6 years—much longer than for conventional seedlings

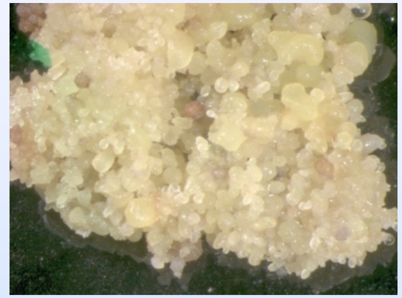
Selected B3F2 trees crossed to get B3F3 seeds

4 months



B3F3 seeds used to start SE cultures

4 months



"Captured" SE cultures

3 months

Somatic embryo production

5 months

Somatic seedlings in vitro

3 months

Somatic seedlings hardened-off to greenhouse

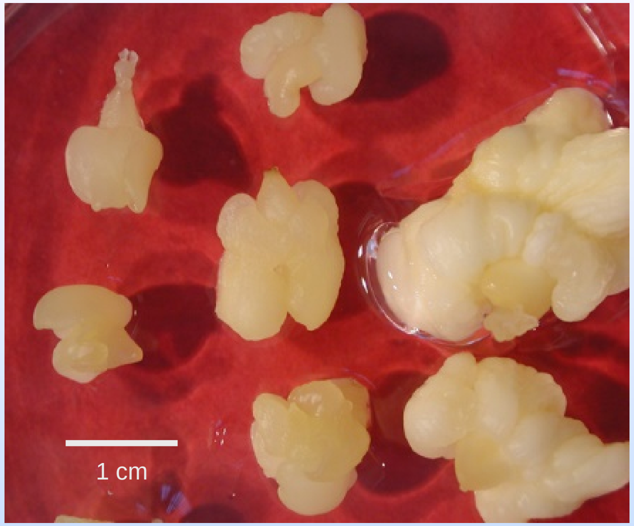
7 months

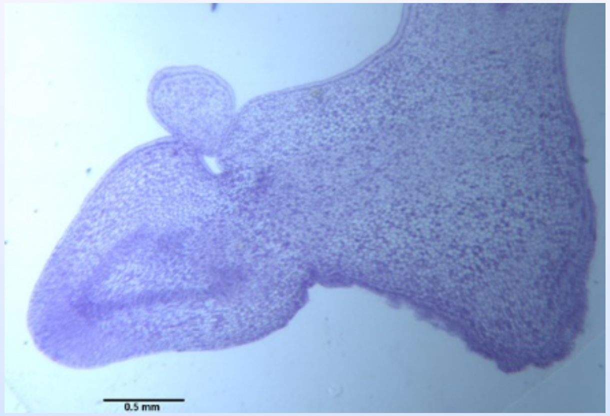
Somatic seedlings moved to shade house

6 months

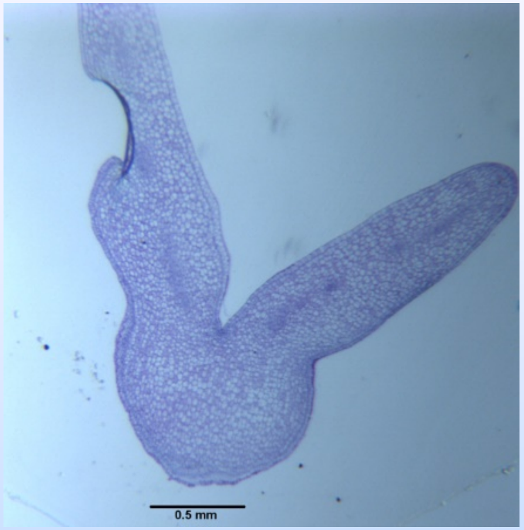
Somatic seedlings planted

Problem: Poor somatic embryo quality





Many somatic embryos lack shoot or root meristems



Solutions:

- Combine SE with micropropagation—harvest shoots from embryos that don't produce taproots and root them to produce plantlets or multiply them
- Combine SE with adventitious shoot production (treat embryos with BA), harvest shoots and root or multiply them

Problem: Acclimatization losses

Solution: a lot of patience

Allowing up to 3 months of very gradual acclimatization results in up to 90% survival

Problem: Somatic seedlings start out much slower than regular seedlings and lack orthotropic growth

Solution: Coppice

Andy Newhouse showing that cut back chestnut somatic seedlings at SUNY-ESF can produce a straight shoot of over 6 feet in 1 season

More potential solutions to poor initial somatic seedling growth

- Coppice somatic seedlings in pots to produce single orthotropic stem
- Target root system growth first—shoot elongation will follow (Ryan McNeill's experiment)

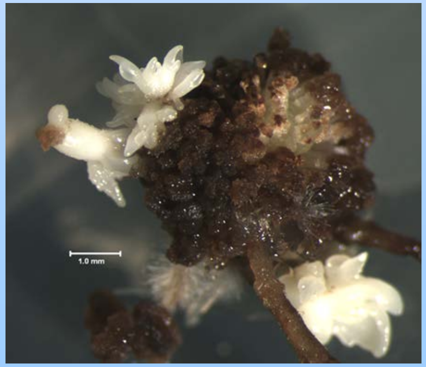


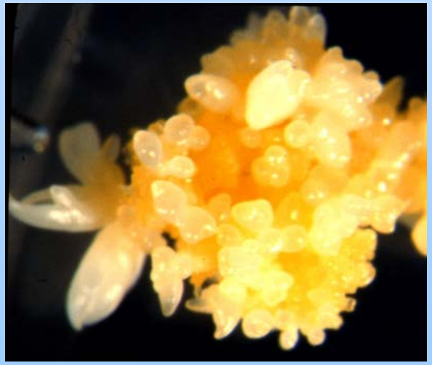
Problem: Cannot clone mature chestnut trees using SE

Potential solutions:

- Test leaf-explant system successfully used with some European oaks
- Test inflorescence system used with sweetgum
- “Gene therapy” (i.e. transform mature tree callus with meristem-promoting genes like WUSCHEL)

Somatic embryos from leaf explant of 80 year old cork oak.
Photos courtesy of Dr. Mariano Toribio, IMIDRA, Madrid, Spain





Somatic embryos from inflorescence explant of 30 year-old sweetgum tree

Current UGA Chestnut Group Personnel

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Technician III
SE culture initiation
& screening

Heather Gladfelter
Postdoc
Transformation &
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Research
Professional III
Cryo-preservation

Ryan McNeill
PhD student
and GA-TACF
Board Member
Chestnut SE

Acknowledgements

The American Chestnut Foundation

Georgia Chapter – TACF

Brad Stanback

Forest Health Initiative

ArborGen Inc.

The American Chestnut Cooperators Foundation - ACCF

Jared Westbrook (TACF)

Fred Hebard (TACF)

Sara Fitzsimmons (TACF)

Marty Cipollini (GA-TACF)

Gary and Lucille Griffin (ACCF)

Bill Powell (SUNY-ESF)

Chuck Maynard (SUNY-ESF)

Andy Newhouse (SUNY-ESF)