

Development and Conservation of *Fraxinus* spp. with Resistance to the Emerald Ash Borer

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Commercial Use of Ash Wood



- Black ash: interior finish, furniture, barrel hoops, traditional snowshoe frames, canoe ribs, woven chair seats, and basketry
- Green ash: crates, boxes, tool handles, furniture, oars, canoe paddles, and for fiber in the manufacture of high-grade paper
- White ash: baseball bats, tool handles, snowshoe frames, boats, furniture, flooring, doors, veneer, and cabinets





Ecological Importance of Ash Trees



- Tolerant of adverse growing conditions in the urban landscape and native forest; hardy to zones 3-9
- Sequester gaseous air pollutants
- Help conserve energy by providing shade
- Provide shelter and food for wildlife in forests and urban areas
- Contribute to the aesthetic pleasure of people





Black Ash

Fraxinus nigra Marsh.



- Important component of wetland forests
- Shade intolerant species; becomes established after disturbance
- Good seed crops produced irregularly (1-8 yr interval)
- Seeds may remain dormant for many years
- Seeds germinate the second spring after being released
- The winged seeds are eaten by birds and mammals
- White-tailed deer and moose feed on the twigs and foliage
- **The wood is valued for paneling, veneer, doors, and flooring**



Objectives

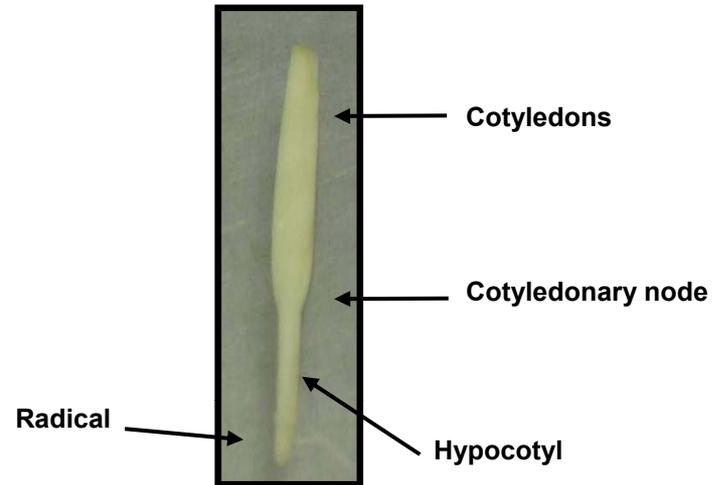
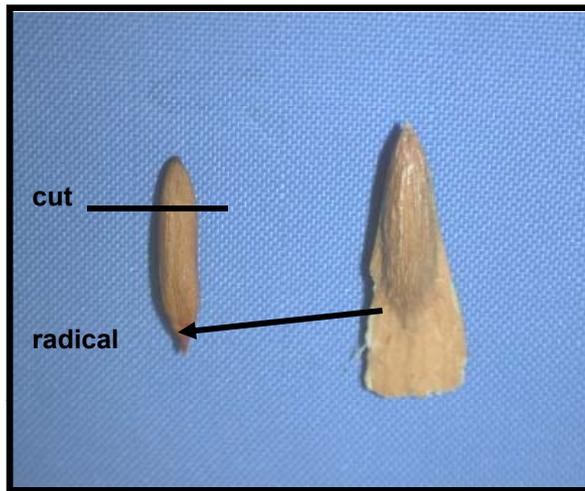


- Develop adventitious shoot regeneration, clonal propagation, and rooting protocols for black, green, and white ash
- Develop an *Agrobacterium*-mediated transformation protocol using molecular marker genes
- Develop a transformation vector (*cry8Da* gene) for the production of ash resistant to the emerald ash borer

Objective 1

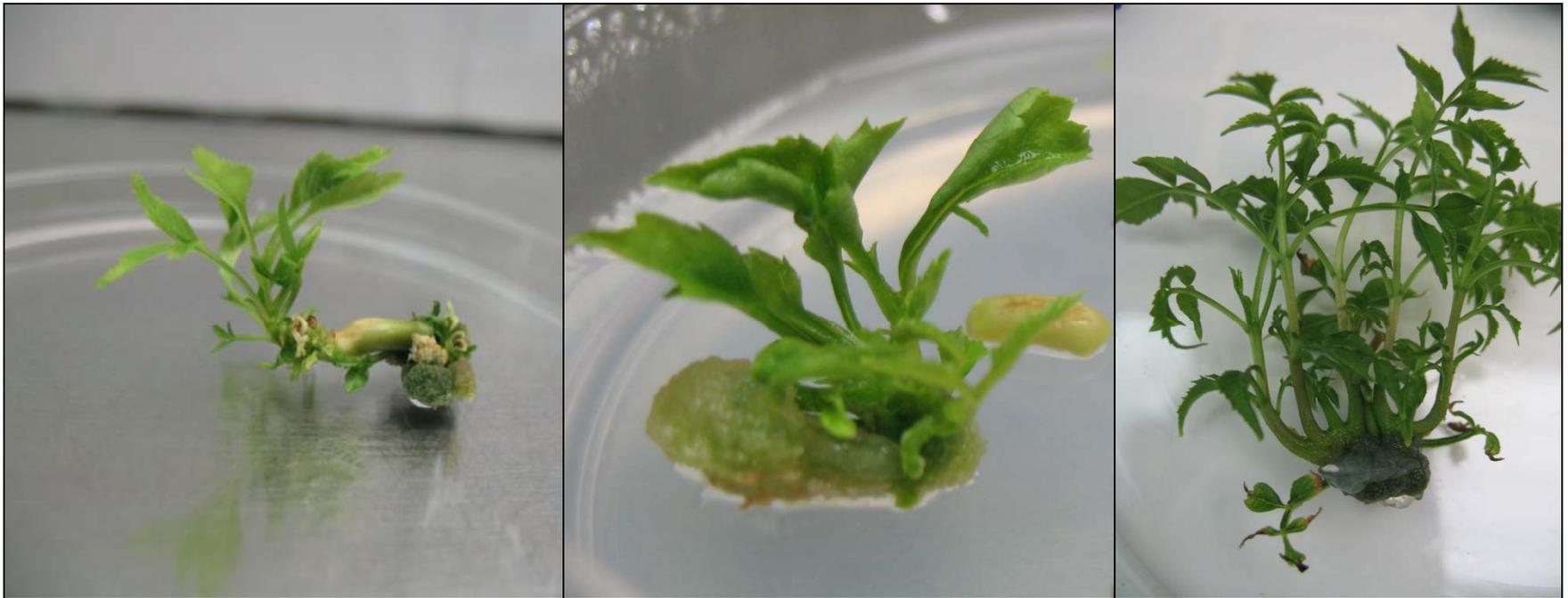
Develop adventitious shoot regeneration, clonal propagation, and rooting protocols for black, green, and white ash

Adventitious Shoot Regeneration



- Mature seeds; pericarp removed; 2-3 mm opposite the radical cut
- Seeds surface disinfected and stored in sterile water in the dark
- Hypocotyl segments excised, cultured on MS medium with 0 to 22.2 μM BA in combination with 0 to 4.5 μM TDZ, 3% sucrose, 0.7% Difco-Bacto agar
- Cultures incubated at 24 ± 2 °C, 16 h photoperiod for 4 weeks

Black Ash Adventitious Shoot Regeneration



Black Ash Rooting



Green Ash Adventitious Shoot Regeneration



- MS medium with 13.3 μM BA plus 4.5 μM TDZ (induction)
- MSB5 with 10 μM BA plus 10 μM TDZ (shoot elongation)
- 75.5 % of hypocotyl segments produced shoots
- 2.7 ± 0.5 shoots regenerated per hypocotyl explant

Green Ash Rooting



- WPM with $4.9 \mu\text{M}$ IBA plus $5.7 \mu\text{M}$ IAA produced the best rooting
- 90 % rooting of shoots
- 3.0 roots per shoot

White Ash Adventitious Shoot Regeneration



White Ash Rooting



Pumpkin Ash

Adventitious Shoot Regeneration



Objective 2

Develop an *Agrobacterium*-mediated transformation protocol using molecular marker genes (three step process)

Transformation via Agrobacterium

(Step 1: gene delivery)

- *Agrobacterium* is a naturally-occurring soil bacterium.
- Unique ability to transfer parts of its own DNA into plant cells.
- In the wild, transfer of a portion of the bacterial DNA (T-DNA) causes rapid plant cell division leading to formation of a tumor.
- In the lab, the tumor forming genes are removed and other genes are substituted. None of the lab strains have the wild genes.
- For *Agrobacterium* to transfer part of its DNA into plants, the bacterium is usually cultured with living, wounded plant tissue.
- After culturing the bacteria with the plant tissues, antibiotics are supplied, eliminating the bacterium from the plant tissue.

Kanamycin Selection (Step 2)



- After DNA delivery, the plant cells are placed on an additional medium, which contains a "selective agent".
- The selective agent is typically an antibiotic, which would normally kill all cells.
- If the introduced DNA also contains a gene for resistance to the antibiotic, only those cells with the resistance gene will survive.
- After sufficient selection, resistant cells will form tissues, which may be capable of regenerating whole plants.
- Whole plants are then regenerated from the single cell or group of cells.

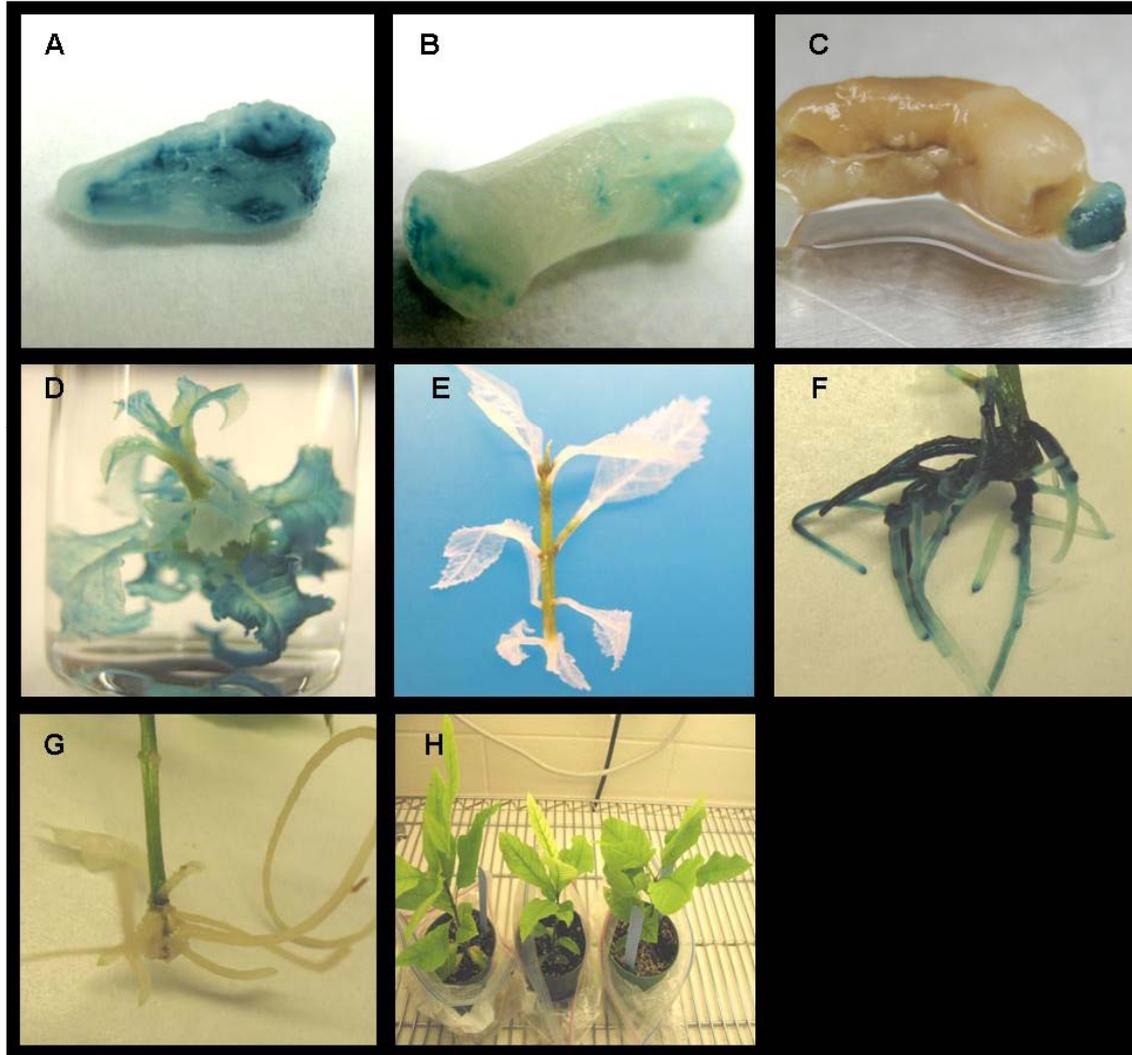
Analysis (Step 3)

- Transgenic plants are recovered and analyzed.
- If everything is done properly, the foreign DNA is integrated and the gene functions as it was designed.
- In many cases, the introduced DNA does not function or functions at low levels.
- To test if the foreign DNA is present, polymerase chain reaction (PCR) is used, which give a plus or minus result (foreign DNA is present or absent).
- To determine if the DNA has integrated as an intact unit and estimate how many copies of the DNA have been introduced, Southern hybridization analysis is used.
- Depending on what genes have been introduced, other analysis are also necessary.

Agrobacterium-mediated Transformation

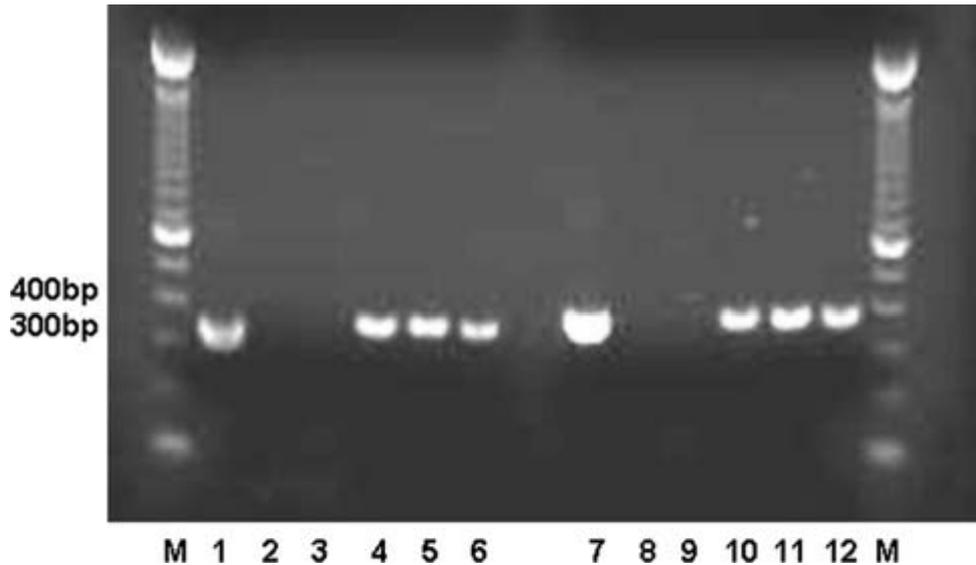
- Hypocotyls excised from 4- to 5-day-old in vitro seedlings, pre-cultured for 1 day on regeneration medium
- *Agrobacterium tumefaciens* strain EHA105 harboring binary vector pq35GR containing the neomycin phosphotransferase (*nptII*) and β -glucuronidase (GUS) fusion gene
- Hypocotyls cultured in 20 ml liquid MS-*Agrobacterium*-pq35GR suspension plus 100 μ M acetosyringone, 90 s sonication plus 10 min vacuum-infiltration
- Co-cultured on regeneration medium plus 50 mg/L adenine hemisulfate plus 10% coconut water, for 2 days in dark culture
- Cultured on regeneration medium plus 20 mg/L kanamycin plus 300 mg/L timentin for selecting transformed cells

Agrobacterium-mediated Transformation

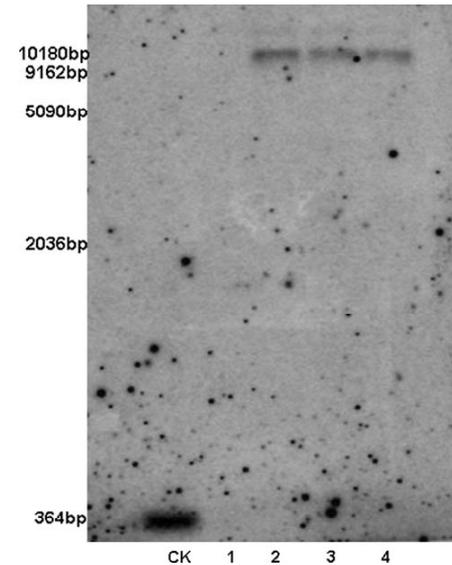


GUS expression and regeneration of transgenic green ash plants

Agrobacterium-mediated Transformation



PCR analysis of genomic DNA isolated from leaves of non-transformed and transgenic green ash plants using primer sets specific for amplification of 364-bp *nptII* gene and 332-bp GUS gene. M 100 bp molecular marker, lanes 1–6 PCR amplification for *nptII* gene, lanes 7–12 PCR amplification for GUS gene. Lane 1 positive control (pq35GR), lane 2 water control, lane 3 negative control (non-transformed plant), lanes 4–6 putative transgenic lines, lane 7 positive control (pq35GR), lane 8 water control, lane 9 negative control (non-transformed plant), lanes 10–12 putative transgenic plants.



Southern blot analysis of transgenic lines of green ash for *nptII* gene. DNA samples (20 µg) were digested with *XbaI*. CK positive control of *nptII*, lane 1 negative control (non-transformed plant), lanes 2–4 putative transgenic plants.

Three transgenic green ash plants acclimatized to the greenhouse.

Objective 3

Develop a transformation vector
(*cry8Da* gene) for the production of ash
resistant to the emerald ash borer

Transformation vector (*cry8Da* gene)

Bacillus thuringiensis (Bt)

- Naturally occurring bacterium
- Produces spores with inclusion bodies (endotoxins)
- Spores and inclusion bodies express insecticidal activity
- Toxin proteins bind to the intestinal wall of the insect, leading to the formation of perforations, and death
- *Cry8Da* toxin confirmed lethal to emerald ash borer adults and larvae (Dr. Leah Bauer, USDA Forest Service)
- Developed genetic construct containing the *Cry8Da* protein for use in genetic transformation of ash; these experiments are underway for developing trees with resistance to EAB



Summary



- Developed an adventitious shoot regeneration, clonal propagation, and rooting protocol for black, green, and white ash
- Developed an *Agrobacterium*-mediated transformation protocol for ash species
- Developed a transformation vector (*cry8Da* gene) for use in the production of putative emerald ash borer-resistant ash

Research in Progress

- Optimizing the *Agrobacterium*-mediated transformation protocol for black and white ash (using marker genes)
- Optimizing the *Agrobacterium*-mediated transformation protocol with green ash using the *Bt* genetic construct
- Developing an adventitious shoot regeneration protocol using leaf explants, so that any mature genotype can be improved
- Investigating other ash species to use with these in vitro systems (pumpkin, blue, etc.)



Acknowledgements



Ningxia Du, PhD (former van Eck scholar)

Rochelle Beasley, Lab Technician-Manager

Kaitlin Palla, former Undergraduate Horticulture student,
van Eck scholar (MS) starting Fall 2010

USDA Forest Service, Northern Research Station,
Hardwood Tree Improvement and Regeneration Center

Fred M. van Eck Foundation for Purdue University

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Fowler Woods State Nature Preserve



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