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## Science and Natural History

# SMALL STEM CHESTNUT BLIGHT RESISTANCE ASSAY

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The current standard method for testing blight resistance in chestnut (Griffin et al., 1983) typically requires trees that are a minimum of 4 cm (~1.57 in) in diameter (Anagnostakis, 1992). Even though chestnut is known for its rapid juvenile growth, it will often require three or more years for a seedling to reach that size. Although the current methods are accurate and can detect intermediate or partial resistance, it would be useful to have a method to determine resistance at a much earlier age. Hebard and Shain (1989) were the first to develop a method to inoculate small stems 7-14mm in diameter (5-18 month old seedlings) that could differentiate resistant Chinese from susceptible American chestnut. Independently, we have developed a similar assay that can distinguish between susceptible American chestnut and resistant Chinese chestnut on stems as small as 3 mm (~1/8 in) in diameter. Some differences in the two techniques include the wounding method in which we use a thin vertical slit as opposed to a circular hole created with a miniature cork-borer, wrapping the inoculation site with Parafilm instead of tape, and removing the inoculum after 5-7 days as compared to leaving the mycelial plug attached. Both methods are useful, but the method described here has the advantage that it can be used on younger seedlings with smaller stems, thereby saving some time. Both methods have the disadvantage that they appear to distinguish only between trees with high levels of blight resistance and those with essentially no resistance and therefore neither method as described is designed to detect intermediate levels of resistance. We currently use highly virulent isolates of *Cryphonectria parasitica,* strains EP155 (ATCC#38755) and EP42 (ATCC#38751), which can be obtained from the American Type Culture Collection (http://www.atcc.org/). A lesser virulent strain SG1 2-3 (Hebard, 2005) might be able to detect intermediate resistance, but haven't tested this yet. Even though this small-stem assay only detects high levels of resistance, it should prove very useful for screening homozygous $F_2$ and $F_3$ trees from the backcross-breeding

program (Hebard, 2005) and for screening trees produced from the transgenic program (Polin et al., 2006). Below is the step-by-step method. If you have any questions, please contact Dr. Powell.

## Method:

**Note:** Pure American chestnut seedlings should be used as a susceptible control and pure Chinese chestnut seedlings should be used as a resistant control and included along side of the trees you are testing.

**1.** Grow test seedlings and controls in pots until the stem diameter about 10cm (~4 in) above the soil line is greater than 3 mm (1/8 in). Depending on how long the nuts have been stratified and the temperature and light intensity of the growth room or greenhouse, it usually takes 12 or more weeks to grow seedlings to the minimum 3 mm diameter. (We routinely grow seedlings in pots in a greenhouse, but the assay might also work with seedlings growing in nursery beds, but this has not been tested.)

**2.** Once the trees have reached the appropriate size, grow *Cryphonectria parasitica* (EP155 and EP42 were used to develop assay) for 3-5 days on Potato Dextrose Agar (PDA) medium.

**3.** Using a very fine-line pen, place a mark 1.5 mm from the tip of a sharp scalpel. We use a Fisherbrand #11 scalpel blade. The mark will be used to ensure your cuts do not go too deep and are consistent from seedling to seedling. (Note: you should also be able to use a razor blade, Exacto knife, or other very sharp, thin cutting tool.)

**4.** With a marker pen, place two marks 5 mm apart (one above the other) on the stems about 10 cm above the soil line.

**5.** Sterilize the tip of the scalpel by dipping in 95% ethanol and then passing the tip though a flame. With the sterile scalpel, cut a thin, 5 mm long wound (from mark to mark) parallel to the stem. This might take a few passes through the same cut to get down to the 1.5 mm depth. (Note: the idea is to make a thin vertical slit in the stem, not to carve out a piece of stem. When done correctly, no wood or bark is removed. This step is critical to a successful assay.)

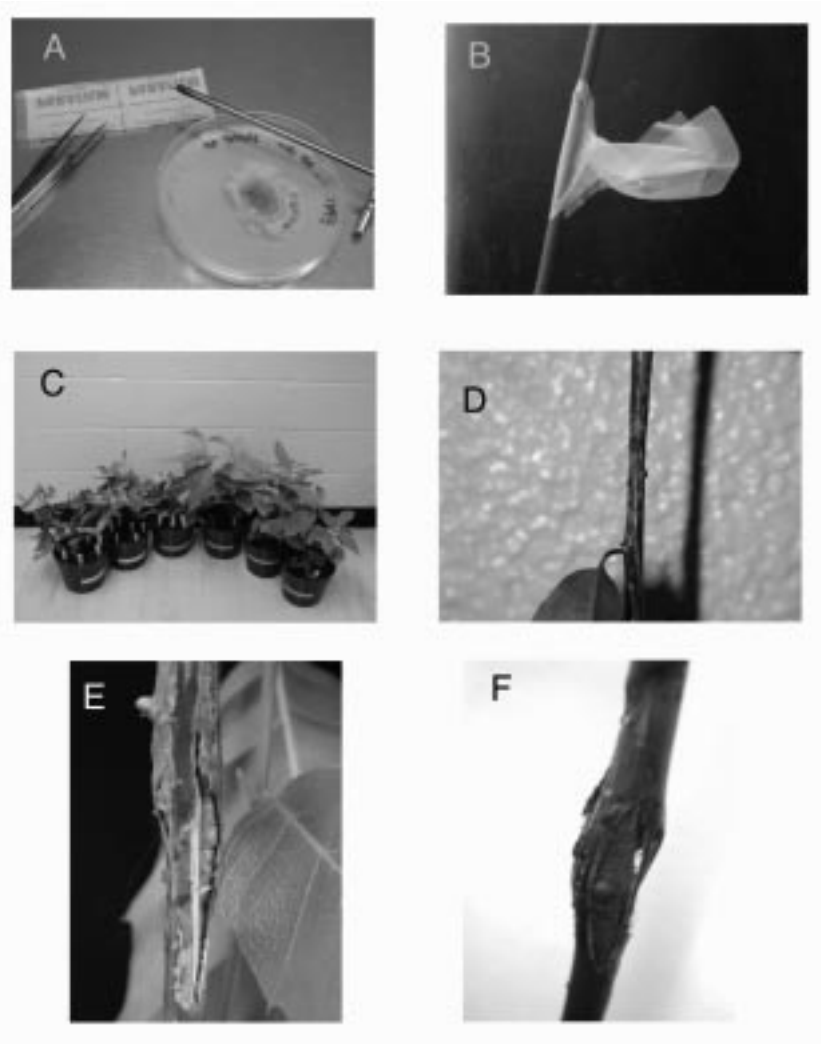


**Figure 1.** Examples of selected stages of the small stem Chestnut blight resistance assay.

A. *Cryphonectria parasitica* prepared for inoculation.
B. Fungal plug placed on wound and sealed with Parafilm.
C. American chestnut (left) and Chinese chestnut (right) after a few weeks.
D. Killing canker on American chestnut seedling stem
E & F. Healing cankers on Chinese chestnut seedling stems

**6.** Aseptically cut many plugs of mycelium from the front edge of the *C. parasitica* colony using a #1 cork borer (~3 mm diameter or 1/8in.) (Figure 1A). (Note: you can also cut the plugs in other ways, such as using the scalpel to cut 3mm size squares.) Remove some of the excess agar from the bottom of the plug to make it easier to handle.

**7.** Take a strip of Parafilm cut to approximately 2.5 cm (1 in) by 10 cm (4 in) and stretch it slightly to make it pliable. Place a plug, mycelium side up, on a strip of Parafilm (Figure 1A). Then carefully place the mycelium against the wound while pulling the parafilm against the stem to make a seal (Figure 1B). Leave enough Parafilm hanging off the opposite side of the stem for easy removal later. This is an important step. Make sure the mycelium is in firm contact with the wound and seal the parafilm around the stem so that the plug does not dry out. (Note: if the mycelium plug slips out of the parafilm, start over with a new plug and a new strip of parafilm.)

**8.** Leave the mycelial plug parafilmed to the wound for 5-7 days. (Note: 3 days was too short in our tests.)

**9.** Remove the parafilm and plug and allow the canker to grow.

**10.** You should see a difference between the controls in about 3-4 weeks (Figure 1C-F). The resistant plants will form callus at the inoculation site and retain their leaves. The susceptible plants will have a sunken canker that completely encircles the stem, the leaves above the inoculation site will wilt, and sometimes new shoots will form below the inoculation site. (Note: We have been able to rescue and reuse many of the susceptible American chestnut seedlings after the assay by cutting off the stem several cm below the canker and allowing a new shoot to form either from an axillary bud or from the root collar.)

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