Chestnut Leaf Inoculation Protocol
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1. Prepare a plate of *C. parasitica* (strain EP155 or SG2-3) on PDA media, subcultured 3-6 days prior to inoculation. Punch plugs around the actively growing perimeter of the culture with a sterile #1 (~3mm diameter) cork borer, or equivalent tubing. Cover plate. Also prepare a gasket-sealed storage tray, such as a Sterilite 16-cup Ultra-Seal Rectangle, for use in step 6. (Trays may be available at Target stores or Amazon.com) Line the tray with >2 sheets of paper towels, moisten paper towels until just damp with dH2O (20-30mL), and cover until needed. Towels should be damp, not soaked.

2. Select leaves from actively-growing stems. Always include blight-tolerant Chinese chestnut and susceptible American chestnut leaves as controls. Collect enough leaves for at least 6 inoculations per leaf type (usually 3-5 leaves). Leaves should be fully expanded and not too soft, but not yet fully hardened. Typically these are the 3rd to 5th leaves below a growing tip. (Younger/smaller leaves are more susceptible to necrosis). Greenhouse leaves are ideal and may be used without cleaning, but field leaves should only be used if they are soaked for 3-5 minutes in mild detergent (i.e. .005% Tween-20) and thoroughly rinsed. Leaves collected in the first half of the growing season tend to be cleaner and easier to measure.

3. Label each leaf with a permanent marker, and mark 1-2 inoculation sites on the midvein. These inoculation sites are 5mm long, and at least 40mm apart. The first starts ~20mm from the petiole, and the second typically starts about half-way along the length of the midvein. Smaller leaves, or those with thinner midveins, should only have one inoculation site.

4. Slice along the midvein at each inoculation site using a scalpel with a sharp #11 blade. Allow the blade to penetrate approximately half the depth of the midvein. Be consistent between leaves.

5. Place a cultured agar plug directly on top of each wound site, with the culture facing the leaf. Press the plug against the leaf firmly enough to be sure it has made good contact with the wound, without breaking or dislodging it. This requires some care, as the small plugs are sticky and difficult to manipulate, but it is essential that the plug remains on the midvein with the fungus firmly touching the wound. Reposition it if it falls off in the next step.

6. Place each leaf, midvein up, in the sealable tray prepared earlier. These trays hold approximately 8-10 leaves each. Keep trays dark (wrap in foil or keep in a dark cabinet), and store at room temperature for 4-7 days (5 days seems ideal).

7. Measure length (along midvein) of necrosis visible on the top (adaxial) leaf surface. (Optional: also measure width to estimate area.) Multiple inoculations on leaves from a single tree or clonal line should be averaged and statistically compared to the controls.

Notes: Leaf age, tray moisture, tray type, temperature, light, length of incubation, leaf type, fungal strain, wound size, and other factors all may affect the outcome of this assay. Therefore, measurements should be interpreted as relative to controls, and results from assays performed under different conditions should not be directly compared.