Protocol for isolation of *Agrobacterium* from herbaceous plant material
Oregon State University Plant Clinic
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This is the protocol used by the Oregon State University Plant Clinic. Because avirulent agrobacteria can be isolated from nearly any type of herbaceous perennial, isolation is not sufficient to show causality. Inoculations must be done to confirm that the isolate is virulent. This is especially important when ambiguous symptoms are present.

We use two types of semi-selective media with each isolation, as isolates may grow on one and not the other.

This is a lengthy process, usually taking a minimum of 3 weeks, not including the time involved with inoculations. It is not sufficient to identify as *Agrobacterium* growth on the semi-selective media, since pseudomonads will also grow on them and may look similar to *Agrobacterium*.

1. Remove soil from and lightly scrub or wash a piece of affected tissue. Rinse well.
2. Surface disinfect affected tissues 1-3 min (depending on sensitivity of the tissue – less for tender tissues) in 10% household bleach (1 part bleach:9 parts water). If the tissue is tender, do not bleach for more than 1.5 min. If the gall is hard, use the 3 min. Rinse well.
3. Aseptically remove a piece of tissue about 5 mm dia. and place into a tube each of 1A and 2E broth. Sonicate for 60 min.
4. Incubate for 1-3 days at 27º C. Incubate for 5 days if using tissue culture material.
5. Vortex tubes. Use a sterilized and cooled bacteriological loop and streak from each tube onto its corresponding agar medium; e.g. streak from 1A broth onto 1A agar plates. Additionally, spread 100 µl of 10-fold dilutions of the broth. For the longer incubation periods use 10^{-5}, 10^{-6}, and 10^{-7} dilutions of the broth cultures; for shorter incubations use 10^{-3}, 10^{-4}, 10^{-5}. Incubate 3 d at 27º C.
6. For *Agrobacterium*: look for domed, mucoid colonies to transfer. The colonies usually have transparent margins and may have some purple, red (1A) or green (2E) color in the centers.
7. Use the selected colonies to transfer to fresh media for purification. Pick up part of a colony with a sterile loop and shake off bacteria into a tube of 5 ml sterile deionized water or saline (if the pH of your water is very far from neutral). Vortex and streak onto MGY. Incubate 3 d at 27º C. Repeat two more times or until colonies look uniform in size, shape, and morphology. Disregard any colonies on MGY that fluoresce under a black light (366 nm), since these will be pseudomonads. *Agrobacterium* colonies on MGY are white, mucoid, domed, and opaque.
8. Stain 24 hr-old cultures to check for purity and Gram reaction. Check oxidase reaction if Gram negative. *Agrobacterium* is Gram negative and oxidase positive.
9. All putative isolates are then put through Biolog to confirm identity. To check for pathogenicity, inoculate onto *Bryophllum daigremontianum*.

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