Title: Nutrient media for determination of sexual reproduction of *Cryphonectria parasitica*.

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Summary:

The most widely used method for determination of sexual compatibility between two isolates of *Cryphonectria parasitica (C.p.)* involves co-inoculation of the strains into dormant American chestnut stems. The method is time-consuming (requiring an average 8-10 weeks for observable perithecial necks of the fungus in chestnut bark) and needs a ready supply of suitably-sized dormant stem pieces. The objective of this project was to find an artificial laboratory medium that would facilitate sexual reproduction in *C. parasitica* in a timely manner.

Eighteen different media formulations, based on their ability to elicit spore production in other fungi, were evaluated in this experiment. Ten *C.* parasitica isolates, representing five known mating type 1 (Mat1) and five mating type 2 (Mat2) strains were paired in various combinations to evaluate candidate media. Crosses involved one isolate being the recipient ("female") of donor conidia ("male"). The crosses were then reversed, so that the former male donor became the female recipient. Treatments were replicated with a minimum of 4 or 5 plates. Crosses were inspected several times per week for the appearance of visible perithecial necks. When black perithecial necks were discernible, several perithecia were teased from the mycelium and evaluated for the presence of viable ascospores.

Of the 18 media formualtions tested in this study, five induced the development of at least some perithecia. One of these, potato dextrose agar (PDA) amended with chestnut bark extract, was successful in eliciting rapid production (average 40 d) of perithecia with ascospores in 18 of 52 petri plates. This formulation, however, still relies on the availability of chestnut bark. The most successful medium was Leonian agar amended with 1 percent "light" (diluted with water to 60 percent) coconut milk, purchased from the Asian foods section of grocery stores. This preparation supported sexual reproduction in 25 of 29 crosses attempted. Eighty-one of 143 plates (56.7 percent) yiekded at least some perithecia in as few as 13 days. Four of 29 crosses (representing a total of 20 plates) never produced perithecia, most likely because some isolates appear to exhibit female sterility. Leonian agar amended with 1 percent "light" coconut milk is a useful alternative to chestnut stems or bark in determining sexual reproduction by *Cryphonectria parasitica.*

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The results of this project were reported at the annual NE-1033 Chestnut Cooperators Meeting in September in LaCrosse, WI.

Manuscript preparation is under way to send results of this experiment to a refereed journal, most likely *Mycologia.*

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Nutrient media for the determination of sexual compatibility of *Cryphonectria parasitica* isolates.

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Project Description:

Since the accidental introduction of the chestnut blight fungus into North America about 110 years ago, and the subsequent spread of the pathogen throughout the natural range of the American chestnut, genetic diversity has apparently increased in *Cryphonectria parasitica* (Milgroom and Cortesi, 1999). The flow of genetic information between/among different populations of the pathogen depends on two factors: vegetative compatibility (VC), which determines whether hyphae of two different strains can fuse; and sexual compatibility, in which opposite mating types produce sexual spores (ascospores). Since sexual reproduction results in significant potential genetic recombination, it is often useful to determine the distribution of both VC types and mating types in a particular population of the pathogen. A complicating factor in determining these characteristics in a given canker, or a given tree, or a given locale, is that vegetatively incompatible strains may still be sexually compatible (Kuhlman and Bhattacharyya, 1984; Marra and Milgroom, 2001).

The currently most widely used technique for the determination of sexual compatibility between two isolates of *C. parasitica* involves co-inoculation of the strains into dormant American chestnut stems (Anagnostakis, 1979; Willey, 1980). This method may not work if the two strains are vegetatively incompatible. To overcome this problem, asexual spores (conidia) are first obtained by growing the mycelium on nutrient plates. The conidia are then used to spermatize the asexual

reproductive structures (pycnidia) of the pathogen growing on chestnut stems. The technique is often time-consuming, requiring 8-10 weeks for development of observable perithecial necks in chestnut bark (M. Double, personal communication), and needs a ready supply of suitably-sized dormant stem pieces. While such material may be relatively easily obtained in the eastern U.S., researchers in many parts of the world do not have easy access to dormant stems.

The development of an artificial medium that would support the production of ascospores in a timely manner has been an elusive goal (M. Double, personal communication; M. Marshall, personal communication). Preliminary work at Alfred State College in 2012 utilized 10 different solid media to determine their suitability for production of perithecia that contain viable ascospores. Isolates of *C. parasitica* were obtained from the culture collection of West Virginia University, and included the Mating Type 1 strains "EP-146" (a brown-pigmented isolate found in West Virginia) and "Schomberg" (from Wisconsin); and the Mating Type 2 strains "EP-155" (originally discovered in Connecticut), "Bockenhauer" (from Wisconsin), and an isolate designated as "6-7-1" (from West Virginia).

Two solid media formulations appeared to be at least partially successful in production of perithecia in as little as 3 weeks after spermatization. One of these, potato dextrose agar (PDA) amended with chestnut bark extract, still relied on availability of chestnut material, but 7 of 12 crosses had observable perithecia and viable ascospores 15-28 days after spermatization. The other media, designated as Leonian agar (Tuitte, 1969) produced less consistent results. The initial crosses between the sexually compatible isolates EP 155 and EP 146 resulted in sparse perithecial production in 3 of 5 plates, and no ascospores in the EP 156 x Bockenhauer cross. A second, larger trial resulted in 2 of 6 EP155 x EP 146 plates exhibiting perithecial necks and ascospores, but none of the other crosses -- representing 20 additional mating pairs in various combinations -- was successful. A review of the protocols revealed slight differences in methodology between the first and second set of trials in the leonian media experiment. These differences can be the starting point for further trials and refinement of techniques.

The development of a simple, time-saving technique for the laboratory media-based identification of mating types of unknown isolates would greatly facilitate studies of the genetic make-up and structure of populations of *C. parasitica.* Toward this goal, I am respectfully requesting $ 1,000 from the Foundation. The funds would be used for the purchase of disposable sterile Petri plates ($ 252 per 500 units), nutrient agar ($ 198.50 per 500 gm), and other media (e.g. PDA @ $ 174.10 per 500 gm). No salaries would be funded from this grant, as unpaid student help is readily available in exchange for college credits in research techniques. Thank you for considering my request.

Literature cited:

Anagnostakis, S.L. 1979. Sexual reproduction of *Endothia parasitica* in the laboratory. Mycologia 71:213-215.

Kuhlman, E.g. and H. Bhattacharyya. 1984. Vegetative compatibility and hypovirulence conversion conversion among naturally occurring isolates of *Cryphonectria parasitica* with broad conversion capacity. Phytopathology 74:659-664.

Marra, R.E. and M.G. Milgroom. 2001. The mating system of the fungus *Cryphonectria parasitica*: selfing and self incompatibility. Heredity 86:134-143.

Milgroom, M.G. and P. Cortese. 1999. Analysis of the population structure of the chestnut blight fungus based on vegetative incompatibility. Proc. Natl. Acad. Sci. 96:10518-10523.

Tuitte, J.F. 1969. Plant Pathological Methods. Fungi and Bacteria. Burgess Publ. Co.

Willey, R.L. 1980. Pathogenicity and sporulation of selected hypovirulent and virulent strains of *Endothia parasitica*. M.S. Thesis. West Virginia University, Morgantown, West Virginia.