Methods for Assessing *Phytophthora ramorum* Chlamydospore Germination[1]

Joyce Eberhart,[2] Elizabeth Stamm,[2] and Jennifer Parke[2]

Abstract

Germination of chlamydospores is difficult to accurately assess when chlamydospores are attached to remnants of supporting hyphae. We developed two approaches for closely observing and rigorously quantifying the frequency of chlamydospore germination *in vitro*. The plate marking and scanning method was useful for quantifying germination of large numbers of chlamydospores over a 7-day period. A method involving time lapse photography of microscope slide cultures was effective for visualizing the process of germination over shorter time periods.

Key words: *Phytophthora ramorum*, chlamydospore, germination, assessment, banana slug

Introduction

Chlamydospores of *Phytophthora ramorum* are believed to contribute to the long-term survival of the pathogen. Understanding the factors that influence chlamydospore germination is necessary for determining their role in pathogen biology and epidemiology. Germination is difficult to assess when chlamydospores are attached to remnants of supporting hyphae. We developed two approaches to observe and quantify the frequency of chlamydospore germination *in vitro*. Colonies formed by hyphal fragments, sporangia, or zoospores were not counted.

Methods and Results

Plate Marking and Scanning Method

To separate chlamydospores from hyphal fragments and other propagules, 8- to 10-week-old broth cultures of *P. ramorum* were blended for 20 seconds, sonicated for 2 minutes, filtered through four layers of cheesecloth, and poured through a sieve (mesh opening = 106 µm). It was then filtered through 20 µm nylon mesh and the chlamydospores scraped off into V8 broth amended with 0.2 percent (w/v) agar. An aliquot of this chlamydospore suspension was spread evenly with a glass rod onto a CMA PARP plate. Using a fine-tipped marker under a dissecting scope, a small dot was made on the Petri dish next to each unattached, non-germinated chlamydospore. Marked spores were checked for germination at 3, 5, and 7 days after plating using a different color ink for each date (fig. 1). The plates were scanned and the scans saved as jpeg files. The open source image processing package FIJI (an ImageJ package) was used to count the dots. With this method we could be certain that the observed colonies were from germinating chlamydospores only, not from hyphal fragments or other propagules.

The plate marking method was used successfully to study how passage of chlamydospores through banana slugs (*Ariolimax columbianus*) affected germination of *P. ramorum* (Parke et al. 2010). Nine-

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[2] Department of Crop and Soil Science, Oregon State University, Corvallis, OR 97331.
Corresponding author: joyce.eberhart@oregonstate.edu.

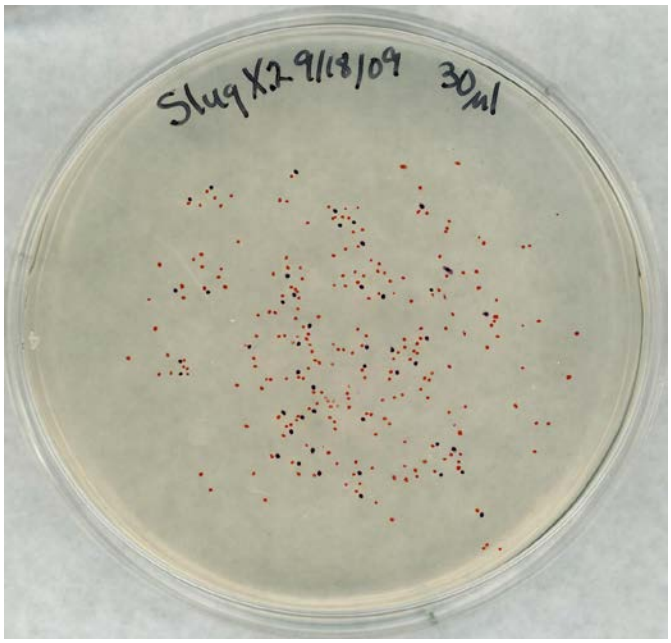

Figure 1—Plate marked with red dots to indicate initial non-germinated, unattached chlamydospores. Black dots indicate those that had germinated by a later date.

to eleven-week-old liquid cultures of *P. ramorum* isolate 4581 were processed as above, and the chlamydospore/V8-0.2 percent agar suspension was offered to banana slugs that had been deprived of solid food for 3 days. The slugs readily consumed the suspension and their feces contained large numbers of chlamydospores (fig. 2). The feces were diluted and spread on CMA PARP plates and germination was followed. Control plates of the same inoculum but without passage through slugs were made to compare germination frequencies. Results were pooled from three separate trials, each with the same three slugs.

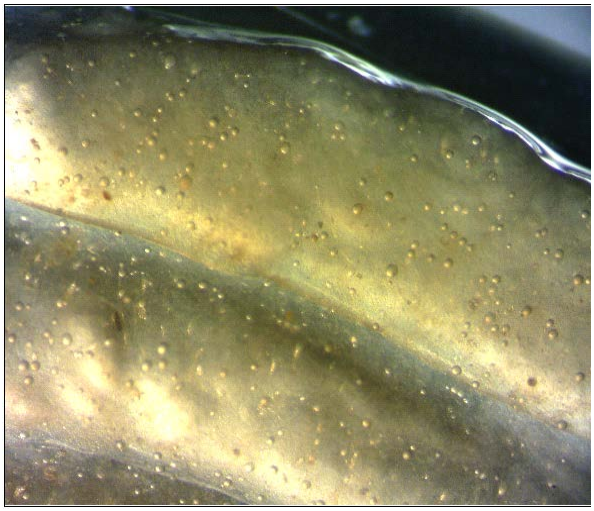

Figure 2—*Phytophthora ramorum* chlamydospores in slug feces.

After passage through slugs, average chlamydospore germination at day 7 was 22 percent, while germination on control plates was 12 percent (p = <0.0001). Results indicate that passage through slugs stimulates the germination of *P. ramorum* chlamydospores (table 1).

Table 1—Percent germination of marked chlamydospores passed or not passed through slugs

	Day 3	Day 5	Day 7
Slugs	17	21	22
Control	9	11	12

This method also gave us the ability to distinguish colonies not growing directly from a germinated chlamydospore. The percent of total colonies arising from marked chlamydospores was significantly different after passage through a slug (83 percent) than the control (68 percent) (p = <0.0001).

Time-Lapse Photography/Microscopy Method

We utilized a combination of time-lapse photography and microscopy to observe the early stages of chlamydospore germination. Chlamydospores scraped off of 2-week-old agar cultures of *P. ramorum* isolate 4581 were separated from hyphae and other structures with a blending/sonication/filtration method similar to the method described above. Chlamydospores were then placed on microscope slides dipped in CMA PAR agar. Slides were observed at 12-hour intervals for 36 hours with brightfield microscopy, using a 425 nm wave length filter. With this method it was easy to distinguish between emerging germ tubes and regrowth from subtending hyphae, allowing an accurate assessment of germination (fig. 3). Observations were discontinued after 36 hours due to hyphal growth obscuring further chlamydospore germination. At 36 hours, approximately 5 percent of the chlamydospores had germinated.

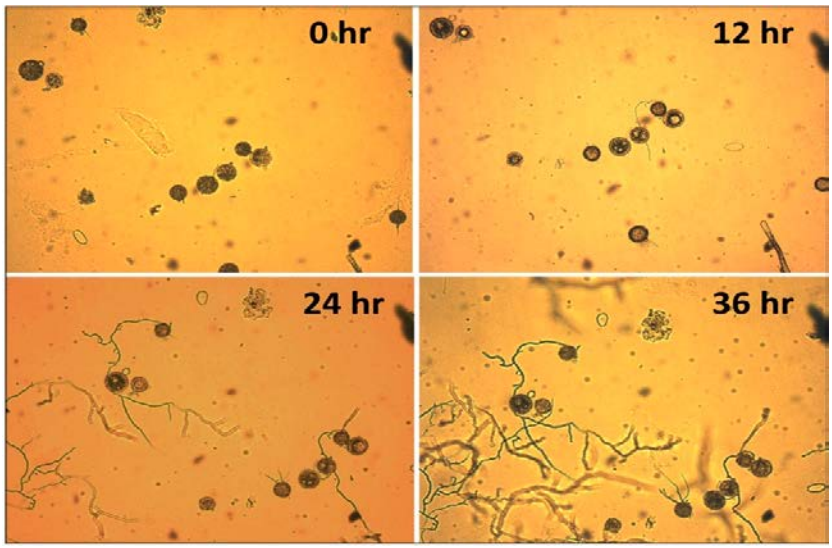

Figure 3—Time sequence of *Phytophthora ramorum* chlamydospore germination at 0, 12, 24, and 36 hours after placement on microscope slides coated with CMA PAR.

Discussion

Both methods were useful for quantifying chlamydospore germination without interference from hyphal fragments or other propagules. Time-lapse photography of microscope slide cultures was well-suited for closely observing the germination process during the first 36 hours. The plate-marking method was useful for monitoring larger populations of chlamydospores over a longer time period.

Literature Cited

Parke, J.L.; Eberhart, J.; Stamm, E. 2010. Stimulation of chlamydospore germination and potential for acquisition and transmission of *Phytophthora ramorum* after passage through the Pacific banana slug *Ariolimax columbianus*. Fifth meeting of the IUFRO Working Party S07-02-09, *Phytophthora* diseases in forests and natural ecosystems. New Zealand Journal of Forestry. 41: supplement (abstract).