Evaluation of *Phytophthora ramorum* in Nursery Crop Tissue Culture Propagation

Robert G. Linderman, Research Plant Pathologist, and E. Anne Davis, Biological Laboratory Technician, USDA-ARS Horticultural Crops Research Laboratory, Corvallis, OR 97330

Corresponding author: Robert G. Linderman. lindermr@science.oregonstate.edu


**Abstract**

*Phytophthora ramorum*, cause of Ramorum blight on numerous woody ornamental shrubs, is a regulated pathogen in the US and internationally. Currently, nurseries are inspected to detect infected plants; however, many plants are propagated by tissue culture nurseries and the behavior of *P. ramorum* in this system is unknown. Pathogen growth and sporulation in propagation vessels containing different multiplication and rooting media, with a range of plants and without plants, was evaluated with regard to pathogen visibility and induction of disease symptoms. Within 2 weeks, the pathogen colonies were visible to the naked eye on all 26 multiplication media and on 9 of 11 rooting media tested (without plants). The appearance of colonies on different media was variable and no sporangia but occasional chlamydospores were produced. The pathogen growth was very visible on multiplication media containing susceptible plants, inoculated plants exhibiting obvious discoloration and mortality. The pathogen was reisolated from terminal shoot tissue and roots of symptomatic plants. Variability occurred in susceptibility of different cultivars of a plant species, in virulence of the two isolates of the pathogen, and in recovery from shoot tissue. We conclude that fungal growth on the media, with or without plants, and symptoms of disease were apparent enough that contaminated vessels would be destroyed.

**Introduction**

The discovery of Ramorum blight, caused by the pathogen *Phytophthora ramorum*, on a wide range of ornamental plants in US nurseries as well as nurseries and landscapes in several European countries has resulted in the disease and the pathogen being regulated by quarantine to prevent geographic dissemination (10). Currently, nurseries are inspected to detect infected plants and thereby prevent dissemination of the disease geographically (6). Numerous plant species have been shown to be susceptible in nurseries and landscapes (1,2,5,7,9,11,12). Many of those plant species and cultivars are propagated vegetatively in tissue culture by commercial nurseries. While it is expected that propagation of plant material under aseptic conditions would preclude infection by fungal pathogens, there was still need to evaluate the behavior of *P. ramorum* in the micropropagation system in order to ensure that contamination and disease symptoms on susceptible plants would be apparent enough that the growth vessels would be discarded, and therefore the pathogen would not be disseminated geographically.

Thus, our objectives were to determine the capacity of *P. ramorum* (both European A1 and North American A2 mating types) to grow and be visible on different tissue culture multiplication and rooting media, and to inoculate a range of plant species and cultivars in tissue culture vessels to determine pathogen visibility, plant susceptibility, the nature of infection, and symptom development over the several weeks of the tissue culture propagation process.
Inoculum Production, Inoculation, and Disease Evaluation

The two isolates of *P. ramorum* used in this study were cultured originally from plants in an Oregon macro-propagation, production nursery. Isolate 03-74-N10-A (N10A) was recovered from rhododendron (*Rhododendron* sp.) and is a North American genotype of the A-2 mating type. Isolate 03-74-D12-A (D12A) (European genotype, A-1 mating type) was recovered from Doublefile viburnum (*Viburnum plicatum* var. *tomentosum* ‘Mariesii’) in the same nursery. Cultures were maintained and stored under refrigeration on agar slants until used, and then were transferred frequently on dilute V8 juice agar plates (30 ml/liter of clarified V8 juice instead of the normal 150 to 200 ml/liter) (5). Sporangia to be used as inoculum were produced on dilute V8 juice agar plates (150-mm diameter), starting from a sporangial suspension spread on the plates that were then incubated in a dark incubator at 20°C for 8 days. Sporangia were removed from the plates by flooding with 5 ml of sterile distilled water and scraping the surface of the agar with the edge of a spatula. The aqueous suspension of cauducous sporangia was then poured into a beaker and gently swirled using a magnetic stirrer.

Sporangia were used to inoculate a range of plant species growing in aseptic tissue culture vessels provided by tissue culture nurseries. Sporangia were pipetted onto single spots on the opposite sides and near the edge of the vessels. Cultures were incubated for 6 weeks under a 12-h light regime in a 20°C growth chamber until evaluated for disease symptoms. Two 50-µl drops, each containing 490 sporangia per ml (N10A) or 730 sporangia per ml (D12A), were pipetted onto opposite sides near the edge of each vessel. The concentration of sporangia (or released zoospores) was determined by plating on PARP medium and counting colonies. Disease symptoms were evaluated visually, using a rating scale of 1 = no symptoms different from non-inoculated control vessels, 2 = mild plant stunting and foliage discoloration but no mortality, and 3 = severe plant stunting, foliage discoloration, and plant mortality. There were three replicate vessels for each plant species/cultivar for each *P. ramorum* isolate, with three replicate non-inoculated control vessels. The experiment was not repeated.

Recovery of the pathogen was evaluated from all inoculated plant species by selecting two shoots from near each of the two inoculation sites, surface sterilizing them in 0.05% (N10A) or 0.10% (D12A) sodium hypochlorite solution for 2 min, followed by rinsing in sterile distilled water. In a preliminary test, we determined that sporangia of the two isolates had different sensitivity to sodium hypochlorite. We wanted to eliminate any leaf surface sporangia, but not kill propagules from internal tissue. The shoots were severed approximately 1 cm up from the agar surface, and cut into 1-cm sections representing the lower, middle, and top portions of the shoots. The sections were plated onto PARP selective medium (3) and colony development was recorded after 7 days. Roots of some plants such as birch formed in the multiplication media and exhibited root rot symptoms compared to the non-inoculated controls. Those roots were similarly surface sterilized and plated on PARP medium.

Infection and Symptoms on Tissue Cultured Plants

Among the thirty plant species and cultivars inoculated with sporangia of *P. ramorum* isolates N10A and D12A, some exhibited severe symptoms of infection, including some stem discoloration, leaf necrosis, off-colored foliage such as reddening, and mortality (Fig. 1). Ratings assigned for the combination of symptoms, compared to the non-inoculated controls, indicated that some plant species heretofore not reported as hosts of *P. ramorum* were clearly infected by the pathogen placed in two locations in the tissue culture vessels (Table 1). For some species or cultivars, inoculation with isolate D12A caused more severe symptoms than parallel inoculations with isolate N10A, although the differences were not analyzed statistically. Some plants were clearly not infected as evidenced by both the disease ratings and the isolation attempts on PARP. Infections were confirmed by isolations from plants located near the inoculation sites, and in many cases the pathogen was recovered from the surface-sterilized shoot tip tissue (Fig. 2). However, some cultivars showed no symptoms, yet the pathogen was isolated from shoot tissue (cherry), while in other cases symptoms were apparent but the pathogen could not be isolated.
from shoot tissue (some maple cultivars). In the case of one birch cultivar, where rooting occurred in the multiplication medium, the roots were clearly infected and necrotic, and the pathogen was recovered from surface-sterilized root pieces. This confirms some previous reports (4,8) that *P. ramorum* not only infects and rots the roots, but also infects and grows in the vascular system up in the shoots of the plant. We did not evaluate tissue culture plants growing on rooting media, but expect that the roots of at least those susceptible species or cultivars would be infected and their function impaired. Our results in the tissue culture system also confirm other reports (5,8) of the variability in susceptibility between cultivars within species.

![Fig. 1. Disease symptoms of rhododendron 'Nova Zembla' (A) and hydrangea (B) inoculated with sporangia of *Phytophthora ramorum* A1 and A2 mating types (right and middle, respectively) isolates placed at the black dot at the edge of the medium. Non-inoculated control flasks are on the left.](image1)

![Fig. 2. Isolation of *Phytophthora ramorum* from tissue cultured shoots of rhododendron 'Nova Zembla' (A) or roots of birch (B) on PARP selective medium. Isolations from shoots of rhododendron were from bottom, middle, and top sections (bottom to top of photo) after surface sterilization in either 0.05% (N10A) or 0.10% (D12A) bleach solution for 2 min. Roots of birch for isolations were also surface sterilized. Pathogens grew from internal tissue.](image2)
Table 1. Disease severity ratings and recovery of the pathogen from shoot segments on thirty nursery crop plant species and cultivars 45 days after inoculation of tissue culture vessels with sporangia of isolates N10A and D12A of Phytophthora ramorum.

Plant species - cultivar<sup>x</sup> | Disease rating<sup>y</sup> | Recovery on PARP<sup>z</sup>  
--- | --- | ---  
| | D12A | N10A | D12A | N10A  
Birch – 1 | 2 | 1 | 17 | 0  
Birch – 2 | 3 | 3 | 14 | 11  
Birch – 3 | 2 | 2 | 17 | 22  
Birch – 4 | 2 | 3 | 14 | 25  
Red Maple – 1 | 1 | 1 | 0 | 0  
Red Maple – 2 | 3 | 3 | 0 | 0  
Red Maple – 3 | 3 | 1 | 0 | 0  
Red Maple – 4 | 1 | 1 | 8 | 11  
Red Maple – 5 | 2 | 1 | 22 | 8  
Red Maple – 6 | 1 | 1 | 0 | 0  
Red Maple – 7 | 2 | 2 | 25 | 22  
Lilac – 1 | 2 | 2 | 64 | 44  
Lilac – 2 | 2 | 2 | 28 | 11  
Lilac – 3 | 3 | 3 | 11 | 25  
Lilac – 4 | 3 | 2 | 17 | 0  
Lilac – 5 | 2 | 2 | 36 | 6  
Lilac – 6 | 3 | 3 | 11 | 14  
Lilac – 7 | 1 | 2 | 6 | 33  
Linden | 3 | 3 | 31 | 0  
Redbud | 3 | 3 | 14 | 17  
Hydrangea – 1 | 2 | 3 | 39 | 64  
Hydrangea – 2 | 3 | 3 | 42 | 89  
Amelanchier | 3 | 3 | 11 | 22  
Elm | 3 | 3 | 6 | 31  
Cherry | 1 | 1 | 11 | 78  
Crabapple | 3 | 2 | 25 | 53  
Blueberry – 1 | 1 | 2 | 17 | 8  
Blueberry – 2 | 2 | 1 | 0 | 0  
Blueberry – 3 | 3 | 2 | 44 | 8  
Rhododendron | 3 | 3 | 86 | 78  

<sup>x</sup> Cultivar names withheld to protect the identity of the nurseries who donated test plants.<br><sup>y</sup> Rounded averages of disease ratings made from 3 replicate vessels of tissue cultured plants 45 days after inoculation with P. ramorum, using the rating scale: 1 = no symptoms different from non-inoculated control vessels; 2 = mild plant stunting and foliage discoloration but no mortality; and 3 = severe plant stunting, foliage discoloration, and plant mortality.<br><sup>z</sup> Isolation percentages are the averages of 4 plants per each of 3 replication vessels on PARP selective medium (3).
Growth and Sporulation of Pathogen on Tissue Culture Media

Sporangia of both isolates N10A and D12A were inoculated at two opposite sides of tissue culture vessels onto the agar surface of 26 multiplication media and 11 rooting media with different formulations. Two 50-µl drops, each containing 670 sporangia per ml (N10A) or 950 sporangia/ml (D12A), were pipetted onto opposite sides near the edge of each vessel. Growth of the pathogen was evaluated after 3-weeks incubation at room temperature. Both isolates grew (from sporangia and/or released zoospores) and were distinctly visible to the naked eye on all multiplication media and nine of 11 rooting media. The media not supporting growth of the pathogen had been amended with an anti-microbial agent (Plant Preservative Mixture, PhytoTechnology Laboratories, LLC, Shawnee Mission, KS) not included in the other media.

In a second test, sporangia were plated on 17 of the original 26 multiplication media and 8 rooting media poured into Petri dishes. Concentrations of both isolates were standardized to apply 6 to 7 sporangia in 100 µl of sterile water to each plate. Colony morphology, vegetative growth, and production of sporangia and chlamydospores from those plates were evaluated microscopically 7 days later, at which time the number of colonies per plate ranged from 1 to 28 for N10A and 0 to 39 for D12A. Vegetative growth varied among the media, from expanding colonies to colonies with more restricted growth (Fig. 3). Colonies were either on the agar surface, submerged into the medium, or both. Sporangia were not produced on any medium, but chlamydospores were produced on some media.

Conclusions

The results of these studies lead to the conclusion that if the unlikely contamination of the tissue culture system by *P. ramorum* should occur, the growth of the pathogen would be obviously visible within 2 to 3 weeks on any multiplication or rooting media used by different micropropagation nurseries, and those contaminated vessels would be immediately discarded. Destruction of containers with fungal or bacterial contamination is normally a weekly process, looking for any fungal or bacterial contamination. Discovery of contamination likely would occur during the multiplication phase before transfer of plants to the rooting media. Furthermore, contaminated vessels growing susceptible plants, such as rhododendrons, would show visible disease symptoms during the multiplication or rooting phases, prompting destruction of those vessels. Our research indicated that micropropagation nurseries would visually detect contamination and disease symptoms caused by *P. ramorum*, and therefore would not likely disseminate the pathogen geographically.
Acknowledgments and Disclaimers

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Literature Cited