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The effect of salinity on the survival, growth, sporulation and infection of *Phytophthora ramorum*

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ABSTRACT

Phytophthora ramorum has been found in waterways outside infested nurseries, but little is known about its behavior in water. This study examined the effect of salinity on survival, growth, sporulation, and infection. *P. ramorum* survival and growth was negatively correlated with salt concentration (range of 0 −45 g l^{-1}), but showed a level of tolerance even at 45 g l^{-1}. No sporangia were observed in cultures with higher than 20 g l^{-1} of salt and zoospores were not released from sporangia above 14 g l^{-1}. Water sources with different salinity were used to understand the environment where *P. ramorum* can survive and infect host material. Water from natural bodies and water amended with different salt concentrations were added to *P. ramorum*-infested sand and baited with rhododendron leaf disks. Infection decreased with increasing salt concentration and increased with higher initial concentration of *P. ramorum*. This research helps to better understand the effects of water quality on survival and infectivity of *P. ramorum*, expanding the potential survey range.

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1. Introduction

Phytophthora species are grouped in the Oomycota and are often referred to as water molds (Blackwell, 1944). As this name reflects, oomycetes are often associated with moisture and, in general, free water highly favors *Phytophthora* spp. infection of plants. Although the vast majority of known *Phytophthora* spp. are plant pathogens, some recently discovered and identified species have been proposed to have a saprotrophic lifestyle as a rapid colonizer of leaf litter or the very least opportunistic pathogens (Brasier et al., 2003; Hansen et al., 2012). A majority of those species have been found in riparian ecosystems. However, very little is known about their survival and proliferation in this environment.

Phytophthora ramorum is a pathogen that persists and spreads on a wide range of ornamentals. It has been found in irrigation ponds and ditches within nurseries (Jeffers et al., 2010), waterways outside of nurseries (Chastagner et al., 2010), and in streams in forested habitats where *P. ramorum* is known in the natural environment (Sutton et al., 2009). Despite this information, very little is known about the effect of the water characteristics on survival of *P. ramorum* and *Phytophthora* spp. in general. Chandlier et al. (2006) evaluated the effect of water temperature and bacteria on sporulation of *Phytophthora alni* in river water, while Werres et al. (2007) concluded that water temperature was a major influencing factor in production of *P. ramorum* sporangia. In addition, Kong et al. (2012b) found that *P. ramorum* was tolerant to an aquatic environment with a pH range of 5–11. In a related study (Kong et al., 2012a), a higher total salt concentration, as measured by electrical conductivity, stimulated growth and sporulation of *P. ramorum*. One factor in water quality that has not been investigated fully for its effect on *Phytophthora* spp. is salinity, which is related but not the same as electrical conductivity.

In 1990, nine marine *Phytophthora* spp. were reclassified in the genus *Halophytophthora* based upon their differences in morphological and cultural characteristics (Ho and Jong, 1990). This was later confirmed in a study comparing the ITS sequences of genomic rDNA (Cooke et al., 2000). *Halophytophthora* spp., which are closely related to *Phytophthora* spp., have been found primarily in marine ecosystems with only a recent discovery of a freshwater species (Yang and Hong, 2014). *Halophytophthora* spp. have a wide

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tolerance to salinity ranging from fresh to brackish water (Leaño et al., 2000; Nakagiri, 2000). Recently, two *Phytophthora* spp., *Phytophthora gemini* sp. nov. and *Phytophthora inundata*, were isolated from seagrass (*Zostera marina*) in a brackish-saline environment (Man in 't Veld et al., 2011). However, no in-depth studies related directly to the salt tolerance of these two species or any *Phytophthora* spp. could be found. Indirect studies related to disease and soil salinity have demonstrated an effect (Blaker and MacDonald, 1986; Swiecki and MacDonald, 1991), which is often related to salt stress on the plant itself (Snapp et al., 1991). Several studies have demonstrated that although an increase in soil salinity stimulates sporangia formation of different *Phytophthora* spp., zoospore release and activity is reduced (Blaker and MacDonald, 1985; Swiecki and MacDonald, 1991). Some fungi have been found to have a high salt tolerance (Tresner and Hayes, 1971). The level of salt tolerance is important to know for *P. ramorum* to help understand how this pathogen may potentially spread. Understanding the effect of salinity on survival, growth, proliferation, and infection of *P. ramorum* would lead to more knowledge of the potential habitat range and more efficient surveys. Some preliminary results have been reported previously (Preuett et al., 2011).

2. Methods

2.1. Phytophthora ramorum isolates

Six different *P. ramorum* isolates: WSDA-1772 and 5-C (A2 mating type and clonal lineage NA1), PRN-1 and BBA/15 (mating type A1 and clonal lineage EU1), and 09-053 and 05-166 (unknown mating type and clonal lineage NA2) were cultured on 20% clarified V8 agar. The isolates were maintained on autoclaved rye seed for long term storage (Peters et al., 1998).

Mycelium-free chlamydospores of the six *P. ramorum* isolates were produced by a modified method described by Mitchell and Kannwischer-Mitchell (1992) and stored in autoclaved sand at 4 °C as described by Widmer et al. (1998). Five agar plugs (5-mm diameter), containing actively growing mycelium of each of the *P. ramorum* isolates, were placed separately in 20 ml of 20% sterile, clarified V8 broth in sterile Petri plates (100 mm diameter) and stored at 20 °C in the dark. After 4 weeks, the mycelia and chlamydospores were transferred to a blender cup containing 50 ml of sterile water and blended for 20 s. The suspension was mixed with autoclaved masonry sand and stored at 20 °C for at least 4 weeks until the mycelium was no longer viable. The concentration of the chlamydospores in the sand was measured by diluting 5 cm^3 of the infested sand in 95 ml of 0.2% water agar and plating 1 ml on *Phytophthora*-selective medium (PARPH+V8; Ferguson and Jeffers, 1999). The *P. ramorum* colonies were counted after 3 d and the chlamydospore concentration cm^{-3} of sand was calculated based on the average over 10 plates. The sand inoculum was stored at 4 °C for long term storage until it was ready to be used.

P. ramorum sporangia and zoospores were produced by the method described by Widmer (2009). Three agar plugs (5 mm diameter), containing actively growing mycelium of each of the *P. ramorum* isolates, were placed separately in 16 ml of 10% sterile, clarified V8 broth in sterile Petri plates (100 mm diameter) and stored at 20 °C under continuous light (3000 lux). After 5 d, a mycelium-free suspension of sporangia were collected by vigorously shaking the cultures in a sterile conical tube and filtering through two layers of sterile cheesecloth. Zoospores were produced by exposing the sporangia suspension to 4 °C for 1 h and then letting it sit for 45 min at room temperature (20–25 °C). The zoospore suspension was filtered through a 30 μm screen and encysted by vortexing.

2.2. Collection and preparation of water samples

Salt solutions of 6, 14, 20, 35, and 45 g l^{-1} were prepared in the laboratory by dissolving natural white sea salt (La Baleine, Aigues-Mortes, France) in deionized water in a volumetric flask. Salt concentrations of 6, 14, 20, 35, and 45 g l^{-1} were chosen based upon concentration ranges of saline water found in nature and defined by the United Nations Food and Agriculture Organization (FAO, 1992). The saline water ranges for the salt solutions represent moderately saline, highly saline, highly saline, very highly saline, and brine, respectively.

Water samples from Lake Pontchartrain (Slidell, LA; 30° 13′ 07.01″ N; 89° 49′ 23.00″ W), Atlantic Ocean (near Kill Devil Hills, NC; 35° 59′ 08.12″ N; 75° 38′ 19.07″ W), Chesapeake Bay (Norfolk, VA; 36° 56′ 39.86″ N; 76° 13′ 59.87″ W), and York River (Yorktown, VA; 37° 14′ 01.79″ N: 76° 30′ 12.89″ W) were collected by dipping a clean container directly in the water source at the exact points and transferring to a clean glass container that was placed in a cooler until it could be refrigerated. All samples were filtered prior to use through a 5.0 μm, hydrophilic membrane filter using Millipore 47 mm glass vacuum filtering system (Millipore, Inc., Billeria, MA). The water solutions were placed in autoclaved bottles after filtering and stored at 4 °C until they could be used. The pH and conductivity were measured and recorded at room temperature.

2.3. Effect of salinity on growth and sporulation of Phytophthora ramorum

The effect of salinity on the growth of *P. ramorum* was conducted in two different studies. The first study examined mycelium growth as indicated by the dry weight after growing in liquid broth. A sterile 10% clarified V8 broth with final salt concentration of 0, 6, 14, 20, 35, or 45 g l^{-1} were prepared by mixing 3 ml of an autoclaved 20% clarified V8 broth with 3 ml of an autoclaved salt solution of 0, 12, 28, 40, 70 or 90 g l^{-1}, respectively, in a 60 mm diameter Petri plate. An agar plug (3 mm diameter) containing actively growing hyphae from the advancing edge of a *P. ramorum* colony on V8 agar for each of the six *P. ramorum* isolates tested was transferred to each plate. Controls were included for each salt concentration by adding only a V8 agar plug. There were six plates of each isolate for each salt concentration. Three of the plates for each isolate were placed in a 20 °C incubator under continuous light (3000 lux) to induce sporangia production (Widmer, 2010), while the remaining three plates were wrapped and placed in a 20 °C incubator in the dark for mycelium growth. After 1 week, the plates were removed from the incubator and the contents of one plate was poured onto a pre-weighed Whatman™ 1 filter paper (90 mm diameter; GE Healthcare UK Limited, Buckinghamshire, UK) sitting under slight vacuum on a Buchner filter. The filter paper and mycelium were dried under a laminar flow hood for 4 h. The filter paper was weighed and the pre-weight subtracted to obtain the preliminary weight of mycelium. The final weight of the mycelium was obtained by subtracting the average preliminary weight of the corresponding controls for that salt concentration from the preliminary weight of the mycelium. The experiment was conducted twice.

The second study examined the effect of agar plates amended with various concentrations of salt on colony growth. Agar plates were prepared by mixing equal volumes of 40% clarified V8 broth and salt solutions of 12, 28, 40, 70, or 90 g l^{-1} (for final salt concentrations of 6, 14, 20, 35, and 45 g l^{-1}, respectively) with 18 g l^{-1} agar and autoclaving for 20 min. After cooling to 50 °C, the agar slurry was poured into 60 mm diameter Petri plates and allowed to solidify. An agar plug (3 mm diameter), taken from the edge of an actively growing culture of the tested *P. ramorum* isolate, was placed in the center of an agar plate at each salt concentration,