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Abstract approved:

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The chlamydospore is a survival spore produced by 35 of the 75 described species of *Phytophthora*. *Phytophthora ramorum* Werres, de Cock & Man in't Veld, the causal agent of Sudden Oak Death (SOD), produces abundant chlamydospores in artificial culture and plant tissue. The chlamydospore is likely the most important survival structure produced by *P. ramorum* as it is known to exist in wildland, interface, and nursery environments in the United States and Europe. I documented, in varying degrees and combinations, the biology of the chlamydospore in *P. ramorum* grown in V8 juice agar and broth (V8JB) and detached *Rhododendron macrophyllum* (Pacific rhododendron) leaves, related to its development, maturation, and germination. I compared these results to what is known about chlamydospores produced in other *Phytophthora* species. V8 agar- and V8JB-grown *P. ramorum* chlamydospores had the third largest diameter (avg. 52.4, max. 93.0 µm) and fourth thickest wall (avg. 2.3, max. 5.0µm) of chlamydospore-producing *Phytophthora* species. In V8 agar and V8JB, maturity was reached in most chlamydospores by eight to ten days, by which time a septum had formed, chlamydospore expansion had ceased, and thickening of the interior wall had begun. The walls of some chlamydospores continued to thicken (from 0.5 to 4.0 µm) up to 120 days of age in V8JB. Generally, larger diameter chlamydospores tended to have thicker walls in *P. ramorum*. Ten-day-old V8JB-grown chlamydospores of *P. ramorum* germinated at low and variable rates after 24 hours of incubation at 20 C in the dark. Germination was significantly higher on nutrient-rich V8 agar (max. 12.9 %, min. 0.2%) than cornmeal agar amended with antibiotics (max. 3.6 %, min. 0.5 %) or water agar (max. 4.0 %, min. 0.3 %), suggesting that germination was stimulated by the presence of exogenous nutrients. Among chlamydospores from populations of ten-day-old V8JB cultures, the chlamydospores with smaller diameters (42.7 µm) and thinner walls (1.6 µm) were more likely to germinate. It was unclear if there was any significant difference in rates of germination among V8JB-grown chlamydospores aged 10, 90, and 120 days. Ten-day-old V8JB-grown chlamydospores continued to germinate after up to five days of incubation on V8 agar amended with antibiotics (V8ARP). Lesions on Pacific rhododendron detached leaves that had been infected 16 days had more chlamydospores (23 / mm^2) than two-day-old lesions (1 / mm^2) with an estimated total of about 10,000 chlamydospores formed within each full lesion. Mean chlamydospore diameter and wall thickness of 30-day-old leaf-grown chlamydospores

was 47.6 µm and 2.9 µm respectively; this was smaller and thicker than those produced on V8 agar (diameter 50.8 µm , wall thickness 2.1 µm) or V8JB-grown (diameter 54.0 µm, wall thickness 2.1 µm) chlamydospores the same age. The maximum chlamydospore wall thickness was also much thicker in leaf-grown chlamydospores (maximum 8.0 µm) than V8 agar (maximum 4.8 µm) or V8JB-grown (maximum 5.0 µm) chlamydospores. Extracted leaf-grown chlamydospores germinated and formed new colonies at a low frequency over 12 days of incubation on V8ARP. *P. ramorum* was recovered from 100 % of freshly plated leaf lesions, but no recovery of the fungus was seen on cornmeal agar amended with antibiotics (CARP) after leaf lesions were frozen or air-dried for five or ten days. Chlamydospores extracted from leaf lesions frozen 15 days failed to germinate on V8ARP. The experiments presented here represent an important first step in understanding the biology of *P. ramorum* chlamydospores. Further experimentation utilizing these results may lead to effective disease mitigating measures, but more research is necessary to attain this important goal.

Key words: *P. ramorum*, chlamydospore, diameter, wall thickness, germination, *Rhododendron*, recovery.

Biology of Chlamydospores of *Phytophthora ramorum*

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I understand that my thesis will become part of the permanent collection of Oregon State University libraries. My signature below authorizes release of my thesis to any reader upon request.

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TABLE OF CONTENTS

TABLE OF CONTENTS (Continued)

Page

TABLE OF CONTENTS (Continued)

Page

TABLE OF CONTENTS (Continued)

Page

TABLE OF CONTENTS (Continued)

LIST OF FIGURES

LIST OF FIGURES (Continued)

LIST OF FIGURES (Continued)

LIST OF TABLES

LIST OF TABLES (Continued)

Chapter 1

Biology of Chlamydospores of *Phytophthora ramorum*: General Introduction

Aaron Smith

Introduction

Phytophthora ramorum (Werres, De Cock & Man in't Veld) is the causal agent of sudden oak death (SOD) which causes a bleeding girdling canker and subsequent high mortality in *Lithocarpus densiflorus* (tanoak), *Quercus agrifolia* (coast live oak), *Q. kelloggii* (California black oak), and *Q. parvula* var. *shrevei* (Shreve's oak) in coastal areas of California and Oregon (Rizzo *et al*. 2002). It also causes a shoot dieback and foliage necrosis in tanoak and on many native understory woody and herbaceous plants such as *Rhododendron macrophyllum* (Pacific rhododendron), although these forms of the disease are less often lethal to the host. As of February 2007, *P. ramorum* had been shown by Koch's postulates to be a pathogen of 35 plant genera in 20 families (USDA 2007), many of which are important wildland components of natural landscapes across western coastal United States, Appalachia, and throughout Western Europe (Hansen *et al.* 2005).

P. ramorum was first isolated from nursery and garden *Rhododendron* and *Viburnum* plants in the mid 1990s in the Netherlands and Germany and was soon thereafter determined to be the cause of SOD in Marin County, California (Rizzo *et al.* 2002; Werres *et al.* 2001). It subsequently spread in wildland ecosystems to 14 coastal counties of California with profoundly negative ecological effects (COMTF 2006; Rizzo & Garbelotto 2003; Rizzo *et al.* 2005); to Curry County in Southwest Oregon, where there has been an extensive eradication effort in effect for six years to extirpate the fungus from what is now 57 km^2 of infected tan oak forest (Goheen *et al.* 2002; Kanaskie *et al.* 2005); and to parks, garden environments, and ornamental plantings in 12 European countries where the fungus has been isolated from *Rhododendron* spp. and other plant species (Brasier & Kirk 2004; Werres & Kaminski 2005). The imminent threat of spreading the disease to other wildland systems throughout North America, Europe, and other parts of the world, has been the impetus for national and international trade restrictions on the movement of known host plant material outside of and within California and Oregon (Hansen *et al.* 2005; Osterbauer *et al.* 2004; Rizzo *et al.* 2005). Despite aggressive governmental regulatory actions to mitigate the spread of the disease, infected plant material has been shipped from California and Oregon nurseries to 49 states where the fungus was previously not known to occur (Tooley *et al.* 2006) and has been detected from nurseries in plant material shipped across international borders (Hansen *et al.* 2003; Werres & Kaminski 2005). Governmental regulatory agencies and researchers continue to detect the pathogen from nursery stock from CA, OR, WA, and Canada and from locations throughout Europe (Hansen *et al.* 2003; Osterbauer *et al.* 2004; Werres & Kaminski 2005). The realized and potential economic and ecological effects of *P. ramorum* becoming established in wildland ecosystems in which susceptible hosts occur are staggering.

If efforts to slow the spread of *P. ramorum* in wildland areas are to be effective, it is imperative that we have a solid understanding of its biology. We must better understand the basic

biological parameters of the organism related to its capacity for dissemination, establishment, and survival. The results of the experiments presented here contribute to the understanding of these parameters as they relate to the chlamydospores of *P. ramorum.*

Life History of Phytophthora ramorum and Phytophthora spp.

The hyphae (Figure 1 A) of *Phytophthora* species are diploid, hyaline (nearly transparent), initially coenocytic (lacking cross walls) under 100x magnification, but septa may develop in cultures that are several days old. The hyphae of *P. ramorum* are 5-8 µm wide (Werres *et al.* 2001) and have coralloid branching. Colonies on nutrient agar grow in a pattern of concentric rings, having appressed aerial mycelium and a weak rosette-like pattern after 14 days (Werres *et al.* 2001). Hyphal wall thicknesses in *P. parasitica* were measured to be 0.05 µm at the hyphal tip and about 0.2 µm in mature hyphal segments 5 mm back from the tip in a seven-day-old culture (Hunsley 1973), observations in this study indicate mature hyphal wall thicknesses of *P. ramorum* are similar. *Phytophthora* hyphae readily branch in patterns associated with the environment in which they are growing (Ho 1978). Colonies growing on nutrient-rich media are more densely branched. *P. ramorum* colonies grow at an average rate of 2.8 (range 2.6-3.5) mm/24h on carrot agar at an optimum temperature of 20 C (Werres *et al.* 2001). On nutrient-poor media or at lower temperatures the fungus grows more slowly. *Phytophthora* species are not able to grow at matric potentials below – 50.0 bars (Erwin *et al.* 1983). *P. ramorum* is a cold-tolerant species with minimum and maximum growth temperatures of 2 and 27 C (Werres *et al.* 2001). Nutrients and especially sterols are taken up by the fungus and utilized in the formation of new mycelial walls, organelles (Bartnicki-Garcia 1990), and subsequent sporulation (Erwin & Ribeiro 1996). *Phytophthora* spores are formed from expanding hyphal walls (Figure 1 A to B, A to C, and A to F) (Hemmes & Wong 1975). Presumably spore expansion is aided by osmotic pressure from the cytoplasm, which occurs before a septum is formed and it is partitioned from the parent mycelium (Dearnaley *et al.* 1996; Hemmes & Lerma 1985). Following the formation of the septum, parent hyphae are often evacuated of cytoplasm in the immediate vicinity of the septum.

The oospore (Figure 1 B) is the spore type that results from the sexual process of gametangial fusion. Oospores are produced by some species of *Phytophthora* (Erwin & Ribeiro 1996). The formation of an oospore begins with the formation of an oogonium (female gametangium) which is fertilized by the antheridium (male gametangium). In heterothallic (self-sterile) species, like *P. ramorum*, the thalli (singular: thallus) of two mating types A1 and A2, come in physical contact with each other, a pheromone is then produced inducing the formation of the oogonium and antheridium. Upon fertilization, meiosis and karyogamy take place and the resulting spore is termed an oospore. A1 mating types of *P. ramorum* will produce oogonia in the presence of a tester strain of A2 *P. cryptogea*

when the two mating types are grown next to each other across a semi-permeable membrane (Werres *et al.* 2001); however, tester strain crosses are not recombinants of *P. ramorum*. A1 x A2 crosses of *P. ramorum* grown together are recombinant but are often aborted (Brasier & Kirk 2004). In homothallic (self-fertile) species, oogonia are produced under favorable environmental conditions without the presence of an opposite mating type.

The oogonia of *P. ramorum* are terminal, or often laterally sessile, subglobose, 28-38 µm in diameter, with smooth, hyaline walls, and have a wall thickness up to 2 µm on carrot agar (Werres *et al.* 2001). In many *Phytophthora* species, the oospore is the thickest walled spore with wall thicknesses ranging from 1 to 3 µm (Erwin & Ribeiro 1996). The thick walls are thought to allow the spore to survive under adverse environmental conditions such as extremes in temperature, desiccation, and microbial antagonism (Erwin *et al.* 1983). Oospores have been produced in the laboratory in *P. ramorum,* but are not known to occur under field conditions (Brasier & Kirk 2004). In other *Phytophthora* species, oospores will germinate under the appropriate conditions such as blue light, correct age, presoaking treatments, enzyme treatments, temperature treatments, and exogenous nutrients (Erwin *et al.* 1983). Oospores that germinate in a nutrient-poor environment (e.g. water) will only form one or more germ tubes (Figure 1 B to A), whereas germination in a balanced nutrient environment will give rise to one or more germ tubes and sporangia (Figure 1 B to C) (Erwin *et al.* 1983). Oospores of *P. ramorum* have not yet been observed to germinate.

The sporangium (Figure 1 C) is an asexually-produced saclike structure in which the entire protoplasmic contents become converted into zoospores (motile infective spores). Sporangia are produced by most species of *Phytophthora*. It is produced directly from mycelium, on a germ tube from germinated oospores and chlamydospores, and from other sporangia (Figure 1 A to C, B to C, and F to C). Sporangia of *P. ramorum* are abundantly produced on agar, they are single or in sympodia, terminal or lateral after proliferation of the subtending hypha, ellipsoid, spindle-shaped or elongate to ovoid, with rounded or occasionally tapering base, and are considered semipapillate (Werres *et al.* 2001). Their average size is 52 x 24 µm and they are produced on the hyphae or from a short pedicel (Werres *et al.* 2001). Sporangia are produced by the fungus in minimally nutrient environments or in water, under aerated conditions, with high humidity, and with access to sterols (Erwin *et al.* 1983).

In some *Phytophthora* species, the ability to reproduce by means of sporangia and zoospores is considered the main reason that some *Phytophthora* diseases can have such explosive epidemiologies under the appropriate environmental conditions (Erwin & Ribeiro 1996). This is especially true with the aerially dispersed *Phytophthora* species like *P. ramorum*. Sporangia of *P. ramorum* are caducous (deciduous), allowing them to function as a dispersal agent, or propagule, via turbulent dispersal or through rain splash or drip to adjacent or underlying susceptible host plants (Davidson *et al.* 2005; Erwin & Ribeiro 1996). Once sporangia have landed on a susceptible host they can release their

zoospores or germinate directly to infect the host (Erwin & Ribeiro 1996). Sporangia are generally thin-walled with wall thicknesses of about 0.5 µm and so are not resistant to environmental extremes such as desiccation, temperature, and UV light (Erwin & Ribeiro 1996). As sporangia age they are no longer able to release their zoospores, their walls thicken, but may maintain their ability to germinate directly (Figure 1 C to A). Sporangia that germinate in a nutrient-rich environment, in the presence of sugars, amino acids, and optimum growth temperatures will produce only germ tubes (Erwin *et al.* 1983). Sporangia that germinate in nutrient-poor environments, in the presence of sterols, and aerated will either produce more sporangia at lower temperatures or chlamydospores at higher temperatures (Erwin *et al.* 1983).

The zoospore (Figure 1 D) is the asexually-produced, primary infective spore in *Phytophthora* species (Erwin & Ribeiro 1996). Zoospores are reniform and have two flagella which allow them to be motile in free water for hours (Erwin & Ribeiro 1996). In *P. ramorum* zoospores were developed in sporangia in water at 17 C (Werres *et al.* 2001). They are able to travel in free water until they come in contact with a host, upon which they encyst, germinate, and infect the host tissue directly by means of a germ tube (Erwin & Ribeiro 1996). Zoospores are membrane-bound, lacking a cell wall and are very susceptible to desiccation and other environmental extremes (Erwin & Ribeiro 1996). However, cysts form a thin cell wall although this does not allow the cyst to be tolerant of environmental extremes.

The second asexually-produced spore is the chlamydospore, which is produced by some but not all species of *Phytophthora*. Chlamydospores are produced directly from hyphae (Figure 1 A to F) in the light or the dark, in the presence of sterols, with aeration, at higher temperatures than sporangia, and in some species, with low nutrition (Erwin *et al.* 1983). In most *Phytophthora* species chlamydospores germinate with access to exogenous amino acids, although in *P. palmivora* high germination rates are seen in water without the presence of nutrients. Chlamydospores that germinate in low nutrition environments form germ tubes with sporangia (Figure 1 F to C) (Erwin *et al.* 1983), which can release zoospores that can infect host tissue. Under nutrient-rich environments, in the presence of sugars and nitrogen, chlamydospores germinate to form germ tubes and grow vegetatively (Figure 1 F to A) (Erwin *et al.* 1983). Chlamydospores are defined by being swollen hyphal segments delimited from the parent hyphae by a septum (Blackwell 1949).

Hyphal swellings are morphological structures produced by some species of *Phytophthora*, and may resemble chlamydospores, but are not considered to be spores (Erwin & Ribeiro 1996; Stamps *et al.* 1990). They are globose to irregular in shape, usually hyaline, terminal or intercalary, and often in clusters (Erwin & Ribeiro 1996). They are most often seen in water cultures of *Phytophthora* species that produce them (Chitzanidis & Kouyeas 1970). They are differentiated from chlamydospores because they are not delimited by a septum (Blackwell 1949). Wall thickness, in most cases, is less than 0.5 µm and is similar to the thickness of mycelial walls (Erwin & Ribeiro 1996). Their function is

not well understood, but it is supposed that they might serve to perpetuate the fungus (Erwin & Ribeiro 1996).

Figure 1: Life History of *Phytophthora*

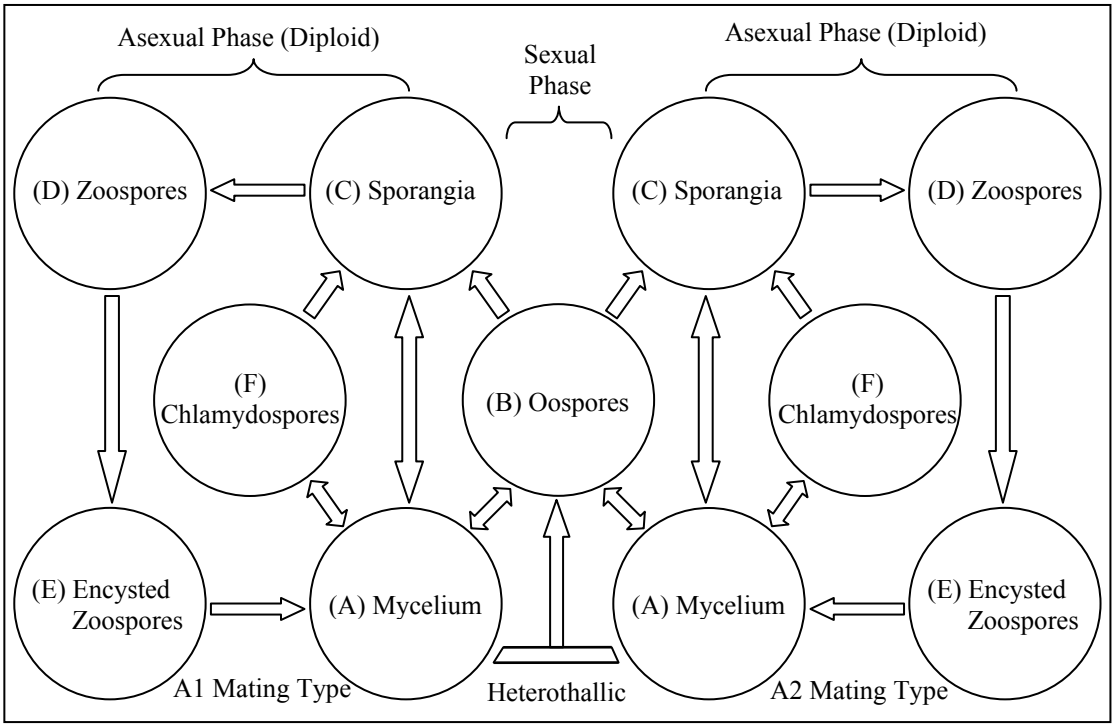

Figure 1 adapted from Erwin & Ribeiro (1996) Figure 1.1 Life History of *Phytophthora*.

Biology of Phytophthora ramorum and Phytophthora spp.

The name *Phytophthora* literally means *phyto* (plant) and *phthora* (destroyer) in Greek. *Phytophthora* species are in the phylum Oomycota, which are taxonomically most closely related to brown algae (Phaeophyceae) and are therefore not true fungi (Erwin & Ribeiro 1996). They are members of the Eumycota. They are commonly referred to as fungi in the informal sense because of the many fungal-like properties that *Phytophthora* species possess. For the purposes of this thesis *Phytophthora* species will be referred to as fungi. *Phytophthora* species differ from true fungi in several ways: the entire thallus is diploid; their zoospores, produced from sporangia, are biflagellate and motile in water; their cell walls contain only a cellulose skeleton and a β-1, 3-glucan amorphous material instead of chitin; they lack the ability to produce sterols, which must be obtained from exogenous sources; and they are also different in several other microbiological ways as well (Erwin & Ribeiro 1996).

Cooke *et al*. (2000) split the genus *Phytophthora* into eight clades based on the criteria of either an aerial (e.g. *P. infestans*) or soil-borne habit. *P. ramorum* is taxonomically most closely related to species in clade eight composed primarily of soil-borne species including: *P. lateralis*, *P. drechsleri*, *P. cryptogea, and P. syringae* (Cooke *et al.* 2000). *P. ramorum* was determined by internal transcribed spacer (ITS) 1 and 2 and amplified fragment length polymorphism (AFLP) analyses to be most closely related to *P. lateralis*, the causal agent of Port-Orford-cedar root disease (Ivors *et al.* 2004; Rizzo *et al.* 2002; Werres *et al.* 2001). All the species in clade eight primarily produce either nonpapillate or semipapillate sporangia (Cooke *et al.* 2000). The color, size, attachment of chlamydospores, and temperature requirements of *P. ramorum* are very similar to *P. lateralis*, although it produces nonpapillate, noncaducous sporangia, whereas *P. ramorum* is the only member of the clade that produces semipapillate, caducous sporangia (Rizzo *et al.* 2002).

P. ramorum is heterothallic with two mating types, the A1 and A2. When the two mating types are grown together, the fungus is capable of producing oospores in plate pairings (Brasier & Kirk 2004; Hansen *et al.* 2003; Werres *et al.* 2001), but the majority of the oospores are aborted and not viable (Brasier & Kirk 2004). No conclusive evidence is available for sexual recombination occurring in wildland settings. With the notable exception of successful plate pairings under laboratory conditions, *P. ramorum* is known to exist only in its asexual state in wildland, nursery, and laboratory settings in North America and Europe (Werres & Kaminski 2005).

P. ramorum is considered to be an exotic pathogen independently introduced to Europe and North America (Ivors *et al.* 2004) and its origin is not yet known (Davidson *et al.* 2005). This conclusion is based on the following evidence: (1) The genetic structure of the pathogen in North America and Europe is homogeneous as determined by ITS (Ivors *et al.* 2004; Werres *et al.* 2001), *cox* II (Ivors *et al.* 2004), AFLP (Ivors *et al.* 2004), and microsatellite (Prospero *et al.* 2004) analyses, although the North American are less heterogeneous than the European isolates (Ivors *et al.* 2004). (2) To date, the two mating types show strong geographic segregation. The A2 mating type occurs almost exclusively in North America, whereas the A1 mating type predominates in Europe (Werres & Kaminski 2005). However, an A2 isolate was recovered from one nursery in Europe and A1 isolates have been recovered from several nurseries in the United States (Hansen *et al.* 2003; Werres & Kaminski 2005). (3) No naturally occurring host resistance has been discovered in susceptible hosts in California and Oregon (Davidson *et al.* 2005), although variation in susceptibility to the pathogen has been recognized among cultivars of the same host species (Tooley *et al.* 2004). (4) The geographic range of *P. ramorum* is limited and considerably smaller than the geographic range of susceptible hosts (Davidson *et al.* 2005).

All *Phytophthora* species are to some degree parasitic (obtain nutrition from another organism), but vary in their range of hosts and ability to cause disease. *P. ramorum* is considered a necrotroph. It kills host cells in advance of its hyphae and then lives on the dying and dead cells. *Phytophthora* species' lifestyles range from aquatic necrotrophs with saprophytic abilities (e.g. *P. gonapodyides*) to aerially dispersed obligate biotrophs (e.g. *P. infestans*) (Cooke *et al.* 2000). Epidemiological evidence from infested wildland ecosystems in California and Oregon strongly suggests that *P. ramorum* is primarily an aerially dispersed organism by means of its caducous sporangia (Hansen 2006, *unpublished*). The limited research on the organism in plant tissue and soil suggests that *P. ramorum* is able to survive at least six months in buried non-sterile soil and plant tissue (Fichtner 2005, *unpublished*; Linderman & Davis 2006; McLaughlin *et al.*, *unpublished*; Shishkoff & Tooley 2004) and that it is capable of infecting roots of *Rhododendron* from infested soil (Lewis *et al.* 2004). It is not yet clear if *P. ramorum* has any saprophytic capabilities or if it must survive periods without living host material.

The Role of the Chlamydospore in Phytophthora ramorum and Phytophthora spp.

Elizabeth Blackwell (1949) was the first to technically define the morphological characteristics and spore types of the genus *Phytophthora* to avoid further confusion of mixed usage of terms among *Phytophthora* workers. In the now classic work "Terminology in *Phytophthora*" she defined the chlamydospore as it occurs in the genus *Phytophthora*;

> "The chlamydospore (Gr. *chlamys, chlamyd-*, a cloak; *sporā,* a seed) is a perennating walled spore which is slowly built up from a portion of the mycelium, more or less swollen with reserves, delimited by a septum if terminal, or septa if intercalary, and with an extra inner, thickened wall layer which in polarized light is more strongly bi-refringent than the walls of the oogonium and oospore. The shape and specialization are variable and it may be no more than an irregular piece of walled mycelium. The types may be grouped as follows:
> 1. terminal and usually spherical; this type might be confused with an old spherical, terminal hyphal swelling or with a resting sporangium or with a parthenogenetic (Gr. *parthenos*, a virgin; *genesis*, origin) oospore but it should be distinguishable from the first by the septum and birefringence of the wall, and from the second by the absence of a papilla, and from the last by the absence of a separate (i.e. the oogonial) wall.
> 2. intercalary and either spherical, or ellipsoid, or irregular. This last type is no more than a piece of resting mycelium and might be so called.
> …The chlamydospore makes a very resistant sort of spore and gives direct germination by a hypha…" (Blackwell 1949).

Blackwell's definition of the chlamydospore, as it occurs in *Phytophthora,* points out several features about the chlamydospore as a distinct spore type having a specialized role in the life history of the organism. (1) It is a perennating (survival) spore with reserves, that is, it contains all the necessary

organelles and energy required to survive adverse conditions and form a new viable organism upon germination. (2) It is slowly built up from a portion of the mycelium with a wall thickness similar to that of the parental mycelium. (3) It is delimited from the parental mycelium by a septum and therefore is independent of the parental colony. (4) It has an extra thickened inner wall which is thicker than the wall with which it first expanded. (5) It is different from a hyphal swelling because of the presence of the septum and thicker wall (more birefringent). (6) It is different from a resting sporangium (thickened walled sporangium, only capable of vegetative germination). (7) It lacks a separate oogonial wall and so, in many species, its wall is thinner than an oospores'. However, average chlamydospore wall thickness is thicker than the oospores produced in *P. ramorum*. These traits taken together form a framework from which the biological significance of the chlamydospore in *Phytophthora* can be better understood.

Chlamydospores are produced by at least 35 of the 75 described species of *Phytophthora*. Some species produce them frequently (e.g. *P. ramorum*) and some produce them very rarely (e.g. only described once in *P. infestans*). Chlamydospore formation can vary greatly depending on the substrate (culture media or plant tissue) in which the species is grown or isolated from (Erwin & Ribeiro 1996). In some species the formation of chlamydospores must be induced through manipulation of nutrient media or environmental conditions. A survey of the literature on chlamydospore-producing species, found 16 species described as infrequent chlamydospore producers, six described as frequent producers, and ten with no frequency qualifier. Isolates of four species were reported to produce chlamydospores frequently while other isolates of the same species did not produce any. Of the species that produced chlamydospores frequently in all known isolates, *P. cinnamomi, P. lateralis, and P. ramorum* were uniquely listed as abundant chlamydospore producers (Erwin & Ribeiro 1996; Trione 1974; Smith 2007, *unpublished*). *P. cactorum*, *P. capsici*, *P. colocasiae*, and *P. nicotianae* (synonym *P. parasitica*) comprised the species that have some isolates that produce chlamydospores and all four of them did so abundantly (Erwin & Ribeiro 1996; Uchida & Aragaki 1985; Erwin & Ribeiro 1996; Erwin & Ribeiro 1996).

Chlamydospore characteristics have been used as a taxonomic character for differentiating some species of *Phytophthora* (Stamps *et al.* 1990). However, because the morphology of chlamydospores does not vary appreciably among species the significance of this character in taxonomy is largely limited to its presence or absence (Erwin & Ribeiro 1996). Some variable characteristics of chlamydospores that have been used in taxonomy include orientation in relation to hyphae (terminal, intercalary, lateral or sessile), wall thickness (< vs >), and size (less than or greater than 35 µm) (Erwin & Ribeiro 1996). *P. cinnamomi* and *P. ramorum* are differentiated from other *Phytophthora* species by the production of numerous chlamydospores and the characteristic morphology of the hyphae (Erwin & Ribeiro 1996; Werres *et al.* 2001). *P. lateralis* is characterized by the production of numerous lateral,

sessile chlamydospores (Erwin & Ribeiro 1996). *P. macrochlamydospora* is characterized by having large (55.0 µm diameter) chlamydospores (Erwin & Ribeiro 1996).

It is widely stated that chlamydospore production in *Phytophthora* is induced in response to an environmental or physical stress. This reasoning is very much in congruence with the pervasive understanding of the chlamydospore as a survival spore; if conditions are not conducive to growth it is a sound ecological strategy to remain dormant. Early work with chlamydospores of *P. cactorum* found that chlamydospores were formed in response to wide extremes of temperature (Blackwell 1943). Darmono and Parke (1990), working with *P. cactorum*, found that chlamydospores would generally not form at temperatures between 8 and 32 C, only sometimes forming at 24 and 28 C, but would readily form in V8 juice broth (V8JB) after incubation for 20 days at 4 C. Bartnicki-Garcia & Wang (1983) posited that asexual and sexual reproduction of *Phytophthora* species are triggered by mycelium starvation. Tsao (1971) reported that chlamydospores of *P. parasitica* developed abundantly when cultures were submerged to a depth of 50 mm in liquid medium, depletion of nutrients and low aeration were considered to be ideal conditions. Tsao (1969) found that lysis of mycelium was invariably associated with the formation of zoosporangia and chlamydospores.

In contrast to the environmental stress theory for chlamydospore formation, many *Phytophthora* species readily form chlamydospores under optimal growth conditions. *P. cinnamomi* forms abundant chlamydospores in agar culture and infected host tissues at ambient temperatures (Rands 1922). *P. lateralis* also forms abundant chlamydospores in many agar media at temperatures between 15 and 25 C (Erwin & Ribeiro 1996). In *P. ramorum,* chlamydospores are abundantly formed in many nutrient-rich media (Carrot piece (Werres *et al.* 2001), Cornmeal, Potato, Malt extract, and V8 agars (author's observation)) and are formed more sparsely in nutrient-poor media (water agar) under optimal growth conditions. Chlamydospores are also abundantly formed by *P. ramorum* growing under optimal conditions in plant tissue (Lewis *et al.* 2004; Pogoda & Werres 2004; Tooley *et al.* 2004). Chlamydospore production is so pervasive in *P. ramorum* that it is not yet known how to grow the fungus in any media, in-vitro or in-vivo, without forming chlamydospores.

It can be argued that the chlamydospore is the most important survival structure available to *P. ramorum*. In nature, only the asexually-produced structures are presently known to exist; hyphae, sporangia, zoospores, and chlamydospores. Among these, chlamydospores are by far the thickest-walled structures and, in other *Phytophthora* species, have been shown to be the most resistant to environmental extremes (Erwin *et al.* 1983). In other species of *Phytophthora*, resting sporangia have been shown to become thicker-walled over time and have some ability to resist environmental extremes (Erwin *et al.* 1983), but this phenomenon has not yet been documented in *P. ramorum*. The apparent absence of thick-walled oospores in nature, the singular presence of thin-walled asexual structures, and

the prolific production of thick-walled chlamydospores that are produced by the fungus very likely emphasize the central importance of the chlamydospore in *P. ramorum* as a survival mechanism.

Chlamydospore formation is considered a trait of more derived species in the Peronosporales of which *Phytophthora* is a member. In particular *P. ramorum* is a species that has morphological traits and a reproductive system that are considered to be highly derived (Gaumann & Wynd 1952); it is heterothallic (Werres *et al.* 2001); it has terminal semipapillate caducous sporangia (Werres *et al.* 2001), which are present in aerially dispersed species (Cooke *et al.* 2000; Werres *et al.* 2001); chlamydospores are present (Werres *et al.* 2001); it has a wide host range (Osterbauer *et al.* 2004; Tooley *et al.* 2004) and specialized virulence system (Manter *et al.* 2005, *unpublished*) present in many pathogens of woody plants; it exhibits degeneration of sexual reproduction (Brasier & Kirk 2004) and an increased emphasis on asexual reproduction (Brasier & Kirk 2004). Brasier & Kirk (2004) demonstrated the very slow production of oospores in *P. ramorum* compared with other *Phytophthora* species and was only able to obtain A1 x A2 oospores by pairing hyphae in which chlamydospores had not yet been produced by the fungus. One explanation given for this is the possible existence of a strong developmental switch that, under the appropriate environmental conditions, triggers the intensive production of chlamydospores and tends to override the sexual system (Brasier & Kirk 2004). It was also suggested that such a mechanism could reflect the unusual ecology of *P. ramorum* being adapted for a tree canopy-inhabiting lifestyle (Brasier & Kirk 2004). A second explanation was that this characteristic reflected the possible degeneration of the sexual system within *P. ramorum* (Brasier & Kirk 2004).

The chlamydospore in *Phytophthora* is capable of initiating explosive epidemiological patterns given the appropriate conditions (Erwin & Ribeiro 1996). In a study with *P. palmivora* chlamydospores used as inoculum in sterile and non-sterile soil, Ramirez (1975) found that 0.5 chlamydospores / g soil were capable of infecting 50 % of *Carica papaya* (papaya) seedlings, whereas 10.0 chlamydospores / g soil caused 95 % host infection after eight days of incubation in moist paper towels and an additional seven days of exposure to infested soil. Shew (1980) observed 32-46 % infection and similar levels of mortality of *Abies fraseri* (fraser fir) seedlings with 10 chlamydospores / g soil of *P. cinnamomi* maintained at 19, 22, and 25 C. In *P. ramorum* wildland epidemiology of the organism is thought to be driven by wind-blown sporangia (Davidson *et al.* 2005), the role of the chlamydospore is not understood. *P. ramorum* was recovered from potting soil substrates amended with an inoculum of sporangia and chlamydospores for up to six months by baiting (Linderman & Davis 2006). Results from work presented here indicate that *P. ramorum* chlamydospores are able to germinate and form viable sporangia and hyphae.

In some species of *Phytophthora* the chlamydospore serves a vital role in the survival of the organism in environmental conditions that are not conducive to vegetative growth. The basic tenets of evolutionary biology dictate that it would be an unsuccessful strategy for an organism to devote a large

proportion of its available resources to the production of structures that are not important to its competitive success; such a strategy would be strongly selected against in the evolutionary process. *P. ramorum* devotes a large portion of its resources into the prolific production of chlamydospores even under optimal growth conditions. It favors asexual chlamydospore production at the expense of producing sexually recombinant oospores, and therefore more genetically diverse offspring, when A1 and A2 mating types are in physical contact. Evolutionary theory would suggest that the chlamydospore in *P. ramorum* confers some evolutionary advantage to the organism and is therefore vital to its ecological success.

The chlamydospore is perhaps the least understood spore type in *Phytophthora*. This is understandable given its varied significance in some species. It is clear, however, that in *P. ramorum* the chlamydospore holds a unique biological significance which must be understood if we are to have a mitigating effect on its establishment and spread in wildland ecosystems. This thesis is a first step towards understanding the biological role of the chlamydospore in *P. ramorum*. The objectives of this research were to: (1) describe the development and maturation process of chlamydospores in-vitro (Chapter 2); (2) investigate some of the conditions necessary for germination of chlamydospores and explore the period of time over which germination occurs in-vitro (Chapter 3); (3) define salient characteristics of chlamydospores that tend to germinate in-vitro and those that do not (Chapter 3); (4) follow the development of *P. ramorum* leaf lesions of *Rhododendron* (in-vivo) (Chapter 4); (5) quantify the number of chlamydospores that develop in *P. ramorum* leaf lesions of *Rhododendron* (in-vivo) (Chapter 4); (6) demonstrate the ability of leaf-grown chlamydospores to germinate and form new colonies (in-vivo then in-vitro) (Chapter 4); (7) explore the ability of the fungus to survive freezing and drying in leaf tissue (in-vivo then in-vitro) (Chapter 4); and (8) compare matured leaf-grown chlamydospores to in-vitro chlamydospores in which development and maturation had been followed (Chapters 2 & 4).

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Chapter 2

Biology of Chlamydospores of *Phytophthora ramorum*: Chlamydospore Development

Aaron Smith

Introduction

An integral component to a better understanding of the biology of the chlamydospore in *P. ramorum* is a solid understanding of its development. There has been relatively little research done on the development of chlamydospores in *Phytophthora,* and what has been done has largely focused on documenting specific stages of development. To date, no published research on chlamydospore development exists for *P. ramorum*. It is important to understand the process of development as it occurs through time, what affects it, when it begins, how it transpires and over what period of time, how it matures, and when it ceases. With this sort of understanding it will be possible to more accurately describe the development of the chlamydospore in *P. ramorum* at any given state of development. Such knowledge can provide a framework which will enable better predictions about what behavior can be expected from a chlamydospore in a given state of development.

Factors Affecting Chlamydospore Formation

Several nutritional factors affect the formation of chlamydospores in *Phytophthora*. *Phytophthora* species do not produce sterols; they must be obtained from exogenous sources. Sterols are taken up by the fungus and used to augment the function of membranes in subcellular organelles such as mitochondria and; they are not used as a carbon or energy source (Erwin & Ribeiro 1996). Growing *Phytophthora* species on media containing sitosterol increases the susceptibility of the fungus to polyene antibiotics such as pimaricin and nystatin, which ordinarily have no effect on members of the Pythiaceae (Erwin & Ribeiro 1996). The production of oospores, chlamydospores, and sporangia in many species of *Phytophthora* is greatly increased on nutrient media supplemented with β-sitosterol (Erwin & Ribeiro 1996). Englander & Roth (1980) found that the addition of sitosterol to V8 agar increased chlamydospore production from an average of 58 chlamydospores / 10 mm^2 agar to 419 chlamydospores / 10 mm^2 agar in *P. lateralis*. In contrast, Darmono & Parke (1990) found that augmenting V8 juice broth with sitosterol did not affect the production of chlamydospores in *P. cactorum*. There are no published reports on the effect of sitosterol on the production of chlamydospores in *P. ramorum*.

Nitrogen also has been reported to affect the production of chlamydospores in *Phytophthora* species. Different sources of nitrogen had varying effects on the production of chlamydospores of *P. palmivora*. Huguenin (1974) reported the greatest chlamydospore production on media containing casein hydrolysate, 108.0 chlamydospores / mm^3 , followed by urea, 48.4 chlamydospores / mm^3, L-asparagine 46.7 chlamydospores / mm^3, ammonium nitrate 44.0 chlamydospores / mm^3, and potassium

nitrate 38.6 chlamydospores / mm^3 after 28 days. A carbon-nitrogen ratio of 30:1 in media was found to be optimal for production of chlamydospores

In some species of *Phytophthora,* chlamydospore formation takes place under conditions of water stress, in others formation is enhanced by high water potential. In *P. cinnamomi*, abundant chlamydospores were formed at water potentials between -1050 kPa to -2010 kPa, few chlamydospores were formed at -2950 kPa, non-viable mycelium and no chlamydospores formed at -3630 kPa in non-sterile sand amended with glucose and yeast (Malajczuk & Theodorou 1979). West & Vithanage (1979) found that decreasing soil moisture stimulated the production of *P. cinnamomi* chlamydospores. in different soil types. Chlamydospore formation increased at water potentials of -300 and -500 kPa in gravel, at -1500 kPa and -30 kPa in soil. In contrast, Tsao (1971) reported that chlamydospores of *P. parasitica* formed abundantly when cultures were submerged in sterile DIH_2O at 25 C for six days and then 15-18 C for two to three weeks. The effect of water potential on chlamydospore formation has not been investigated for *P. ramorum*.

Temperature has differing effects on chlamydospore formation in different *Phytophthora* species. Some species form chlamydospores optimally at temperatures that are warmer than their optimum for growth. Englander & Roth (1980) reported that optimum chlamydospore formation took place at 24-25 C (12-26 C) in *P. lateralis*, which has an optimal growth temperature of 20 C (maximum 26 C) (Erwin & Ribeiro 1996). Chlamydospores of *P. palmivora* were produced optimally at 32 C, also near its upper limit for growth of 35 C (Chee 1973; Waterhouse 1974). *P. cinnamomi* optimally produced chlamydospores at 25 C (18-30.5 C) (Englander & Turbitt 1979), well within its optimal growth temperatures. In contrast to these high temperatures, Huguenin (1974) found that an isolate of *P. palmivora* optimally produced chlamydospores at 18 C (<10-30.5 C). *P. parasitica* also produced chlamydospores optimally at moderate temperatures 15-18 C (<12-30 C) (Tsao 1971). No work on the effects of temperature has been done in *P. ramorum*, although abundant chlamydospores are produced at 20 C, which is also its optimal growth temperature (2-30 C) (Werres *et al.* 2001).

The effect of light on chlamydospore production in *Phytophthora* varies by species. *P. cinnamomi* optimally produced chlamydospores on V8 juice agar plus sitosterol (20 mg/ L) when incubated at 23-28 C under near-UV light (310 to 420 nm region of the light spectrum) (Englander & Turbitt 1979). In contrast, Englander & Roth (1980) found that *P. lateralis* chlamydospore formation was not stimulated by light in V8 juice agar or broth not amended with sitosterol; instead, chlamydospores were produced optimally in the dark and not when exposed to light at several wavelengths, including Blacklight, Blue, and Cool White fluorescent lamps. Darmono & Parke (1990) found that Cool White fluorescent lamps did not affect chlamydospore formation in *P. cactorum*. There have been no formal studies on the effect of light on chlamydospore formation in *P. ramorum*.

Chlamydospore Development and Maturation

Chlamydospore formation takes place in *Phytophthora* by the expansion of the cell wall in a segment of a hypha (Hemmes & Wong 1975). During the expansion phase, the chlamydospore wall is thin (about 0.2 μm) and is about the same thickness as the parental hyphal wall segment from which it is formed (Hemmes & Wong 1975). The expansion phase is thought to be aided by the osmotic pressure of the cytoplasm. Upon full expansion the chlamydospore is delimited from the parent hypha by the formation of a septum (in terminal chlamydospores) or septa (in intercalary chlamydospores). (Blackwell 1949). The time required to initiate and fully expand chlamydospores differs among *Phytophthora* species and varies from two days (e.g. ***P. cinnamomi***, Cahill ***et al.*** 1989, Appendix A) to nine months (e.g. ***P. infestans*** Patrikeyeva 1979, Appendix A).

The formation of a septum marks the beginning of the wall thickening phase of the chlamydospore maturation process. An inner wall is built up on the inside of the chlamydospore, forming a second often thicker layer than the expanding spore wall (Hemmes & Wong 1975). The chlamydospore wall is composed of a β-1, 3-glucan amorphous material on the outside, with an inner thickening of glucan microfibrils that contain electron-dense deposits (Bartnicki-Garcia & Wang 1983).

The wall thickness of the fully developed chlamydospore varies among species of *Phytophthora.* For the purposes of this thesis, thin-walled species are defined as being less than 1.0 μm, intermediate as being 1.0-1.5 μm, and thick as being greater than 1.5 μm thick (Kadooka & Ko 1973). Chlamydospore wall thicknesses for 19 *Phytophthora* species reported in the literature include five species considered thin-walled, five considered intermediate, and nine considered thick-walled by this definition (Peterson 1910; Van der Plaats-Niterink 1981; Erwin & Ribeiro 1996; Erwin & Ribeiro 1996; Smith 2007, ***unpublished***; Werres ***et al.*** 2001; Tsao 1991; Uchida & Aragaki 1985; Islam ***et al.*** 2005; Erwin & Ribeiro 1996; Kadooka & Ko 1973; Dantanarayana ***et al***. 1984; Hemmes & Lerma 1985; Dantanarayana ***et al***. 1984; Erwin & Ribeiro 1996; Cother & Griffin 1973; Cother & Griffin 1974; Hemmes & Wong 1975; Cother & Griffin 1973; Shew & Benson 1982; Erwin & Ribeiro 1996; Erwin & Ribeiro 1996; Ann & Ko 1980; Tucker & Milbrath 1942; Basu 1980; Zheng & Ho 2000; Erwin & Ribeiro 1996) (Figure 3, Appendix A). It is interesting to note that what is considered "thin-" and "thick-" walled in *Phytophthora* chlamydospore literature is not standardized. The definition from Kadooka & Ko (1973) was chosen because it best reflects chlamydospore wall thicknesses as they occur across the entire genus. ***P. lateralis*** is generally considered a thin-walled species, but had the second thickest wall thickness (7.0 μm) reported for the genus (Tucker & Milbrath 1942) (Figure 3, Appendix A). ***P. ramorum*** has the fourth thickest average chlamydospore wall thickness (2.4 μm), but has the thickest maximum chlamydospore wall thickness (8.0 μm) recorded for the genus (Smith 2007, ***unpublished***; Werres ***et al***. 2001) (Figure 3, Appendix A).

The diameters of fully developed chlamydospores also vary by species and have been used as a taxonomic character to differentiate species. Small chlamydospores are considered to be less than 35 µm and large chlamydospores are greater than 35 µm in diameter (Erwin & Ribeiro 1996) (Figure 4). In published reports of chlamydospore diameter for 30 species of *Phytophthora*, 21 species were considered small and nine were considered large (Erwin & Ribeiro 1996; Erwin & Ribeiro 1996; Smith 2007, *unpublished*; Werres *et al*. 2001; Rands 1922; Erwin & Ribeiro 1996; Cother & Griffin 1973; McCain *et al*. 1967; Tucker & Milbrath 1942; Werres *et al*. 2001; Tucker 1931; Frezzi 1950; Gerrettson-Cornell 1989; Erwin & Ribeiro 1996; Peterson 1910; van der Plaats-Niterink 1981; Ann & Ko 1980; Tucker 1931; Frezzi 1950; Waterhouse 1963; Gerrettson-Cornell 1989; Darmono & Parke 1990; Hall 1993; Dastur 1913; Holiday 1980; Erwin & Ribeiro 1996; Dantanarayana *et al*. 1984; Hemmes & Lerma 1985; Katsura 1976; Erwin & Ribeiro 1996; Erwin & Ribeiro 1996; Hildebrand 1959; Tsao 1991; Mchau & Coffey 1995; Islam *et al*. 2005; Tucker 1931; Waterhouse 1963; Gerrettson-Cornell 1989; Erwin & Ribeiro 1996; Mchau & Coffey 1994; Erwin & Ribeiro 1996; Erwin & Ribeiro 1996; Peries & Fernando 1966; Dantanarayana *et al*. 1984; Erwin & Ribeiro 1996; Stamps *et al.* 1990; Chee 1969; Erwin & Ribeiro 1996; Zheng & Ho 2000; Erwin & Ribeiro 1996; Basu 1980; Cother & Griffin 1973; Cother & Griffin 1974) (Figure 4, Appendix A). *P. cinnamomi* has the fourth largest average diameter (41.0 µm) and the largest chlamydospore (135.0 µm) recorded for the genus (Rands 1922; Erwin & Ribeiro 1996; Cother & Griffin 1973; McCain *et al*. 1967) (Figure 4, Appendix A). *P. ramorum* has the third largest average diameter (54.3 µm) and the second largest chlamydospore diameter (93.0 µm) recorded for the genus (Smith 2007, *unpublished*; Werres *et al.* 2001) (Figure 4, Appendix A). It is interesting to note that three of the five smallest chlamydospore species have been described as infrequent chlamydospore producers (two had no frequency information) and all five of the largest diameter species have been described as frequent chlamydospore producers (Erwin & Ribeiro 1996; Zheng & Ho 2000; Erwin & Ribeiro 1996; Basu 1980; Cother & Griffin 1973; Cother & Griffin 1974) (Figure 4, Appendix A, Part 2).

The objectives of the experiments presented here were to observe the development and maturation of the chlamydospore in *P. ramorum* and relate these observations to what is known about these processes in other *Phytophthora* species. This was accomplished by: (1) investigating factors related to chlamydospore formation in *P. ramorum*; (2) comparing rates of enlargement of chlamydospores in cultures grown on V8 agar and V8JB over time; (3) comparing the rate of wall thickening in chlamydospores grown on V8 agar and V8JB over time; (4) providing V8 agar and V8JB culture chlamydospore diameter and wall thickness composition data; and (5) comparing the relationship between chlamydospore diameter and wall thickness.

Methods

Isolate Information

Two *P. ramorum* isolates were used in these experiments; isolate 2027.1 was isolated from a tanoak bark sample in Curry County, OR, latitude 42.09108, longitude -124.27480, on 07/25/2001; isolate P-110 was isolated from toyon (*Heteromeles arbutifolia* (Lindley) Roemer) in Marin County, CA. Both isolates were identified as *P. ramorum* by ITS sequence according to Winton & Hansen (2001). Isolate 2027.1 was used except where otherwise noted.

Chlamydospore Formation

Long-Term Storage (LTS): A long-term isolate storage (LTS) series was prepared every 90 days for isolates 2027.1 and P-110. Each new isolate series was started by growing the isolate on V8 agar (Appendix B) in 60 x 15 mm petri plates sealed with Parafilm® for four days at 20 C in the dark. One five mm^3 plug was cut from the leading edge of the fungal culture and placed on V8 agar in 100 x 15 mm petri plates, sealed with Parafilm®, and grown for seven days at 20 C in the dark. Four, five mm^3 LTS plugs were cut from each culture and placed in each of 100 vials (Nalgene® 1.5 ml Cryogenic Vials, System 100™) containing one ml of autoclaved DIH_2O, sealed with a cap, and placed in a storage box (Nalgene® System 100 Crybox, 1.0/1.5 ml, 10x10 Array, PolyCarb) at 20 C in the dark until used.

Chlamydospore Growth in V8 Juice Broth (V8JB) and V8 Agar Cultures: Chlamydospores were grown in 25 ml of V8JB (Appendix B) in 100 x 15 mm petri plates inoculated with six LTS plugs. Chlamydospores were also grown on V8 agar in 100 x 15 mm petri plates inoculated with one LTS plug and sealed with Parafilm®. V8 agar and V8JB cultures were stored in a crisper box (250 x 100 x 320 mm) and kept at 20 C in the dark until they were used.

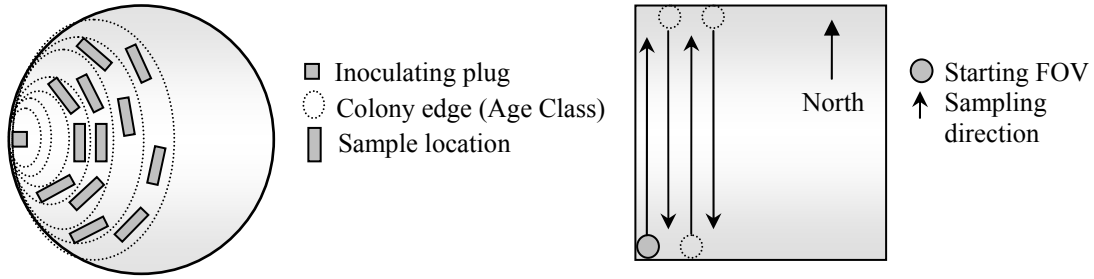
Chlamydospore Photomicrographs: Chlamydospore samples were placed on a microscope slide with a drop of DIH_2O or lactoglycerol and a cover slip. The fungal samples were scanned for desired characteristics using a compound microscope (Zeiss® Axioskop 2) with bright field illumination at 100x magnification. Photomicrographs were taken with a microscope mounted digital camera (Polaroid® Model DMC 1) and processed with digital camera software.

Chlamydospore Maturation

Chlamydospore Maturation on V8 Agar: Wall thickness and diameter of chlamydospores grown on V8 agar for two to 31 days was measured. Three V8 agar plates were inoculated at the edge with a 5 mm^3 plug taken from the margin of a four-day-old colony, then sealed with Parafilm®. (Figure 2 A). The leading edge of colony growth was outlined on the underside of the plate with a fine point permanent marker every two to three days (age class) for 31 days. Ten separate 2 mm^3 samples were cut from the culture at three different locations within each age class (Figure 2 A). The 30 agar samples were placed in a glass vial containing 1 ml of 10% formaldehyde (1ml formalin: 10 ml DIH_2O) for two to 21 days until measured.

Measurements were performed by placing one agar piece on a microscope slide with a drop of lactoglycerol and flattening it with a cover slip. Chlamydospore wall thickness and diameter were measured in sequential 1000x Fields of view (FOV), starting with the southwestern corner of the sample (Figure 2 B), and continuing on additional sample pieces until fifty chlamydospores were measured for each age class.

Figure 2: V8 Agar and V8JB Sampling Pattern



(A) Agar Surface

(B) Agar Sample (V8 Agar)
V8JB Sample (V8JB)

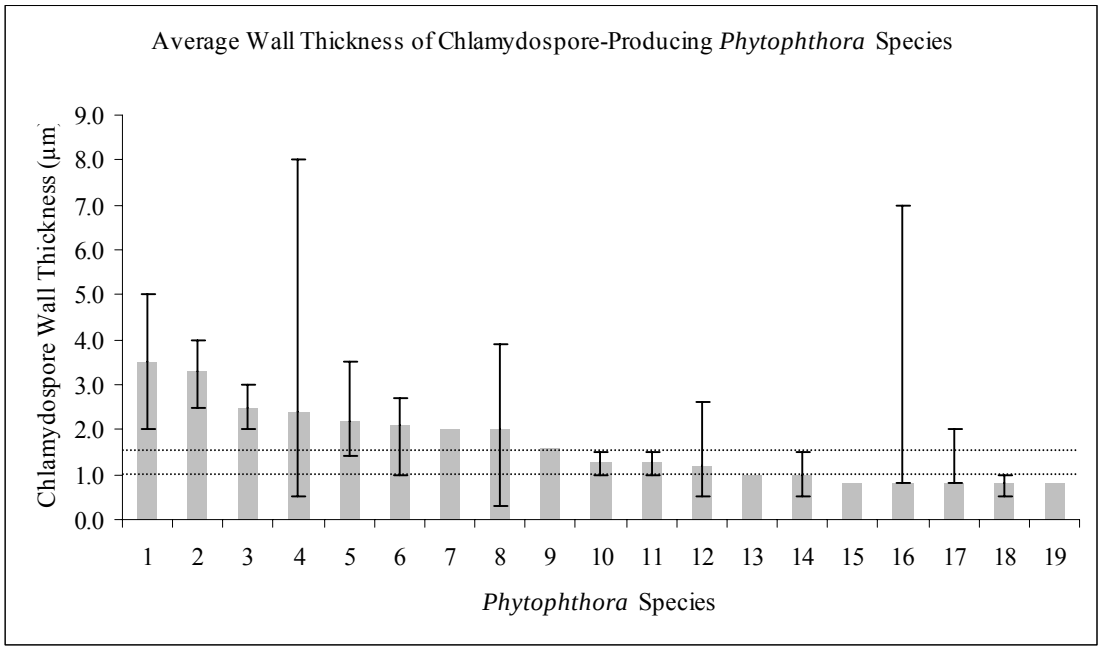
Chlamydospore Maturation in V8JB: In a second experiment, chlamydospore wall thickness and diameter were measured in cultures grown in V8JB for 10, 20, 30, 60, 90, and 120 days (Age class). Sampling methods were similar except three samples were extracted from each culture and placed in formaldehyde for one to five days until sampling, at which time the 50 chlamydospores were measured in one to two samples (Figure 2 B). The experiment was performed once and had three replications of one V8JB culture age class.

Statistical Analysis of Chlamydospore Maturation on V8 Agar and V8JB: Mean chlamydospore wall thickness and diameter were calculated using S-plus® 7.0 for Windows (©1988, 2005, Insightful Corp.) statistical software, by calculating the mean of the measured wall thickness and

diameter values, the associated range of values were constructed with minimum and maximum values. Percentage of chlamydospores in wall thickness and diameter classes were calculated by dividing the number of chlamydospores in a wall thickness or diameter class by the total number of chlamydospores in an age class and multiplying the product by 100. The relationship between chlamydospore diameter and wall thickness was calculated by adding a trend line with its associated r^2 value and formula to a scatter plot of wall thickness and diameter values recorded in the chlamydospore maturation on V8 agar and V8JB experiments. Graphs were created with Microsoft® Excel 2002.

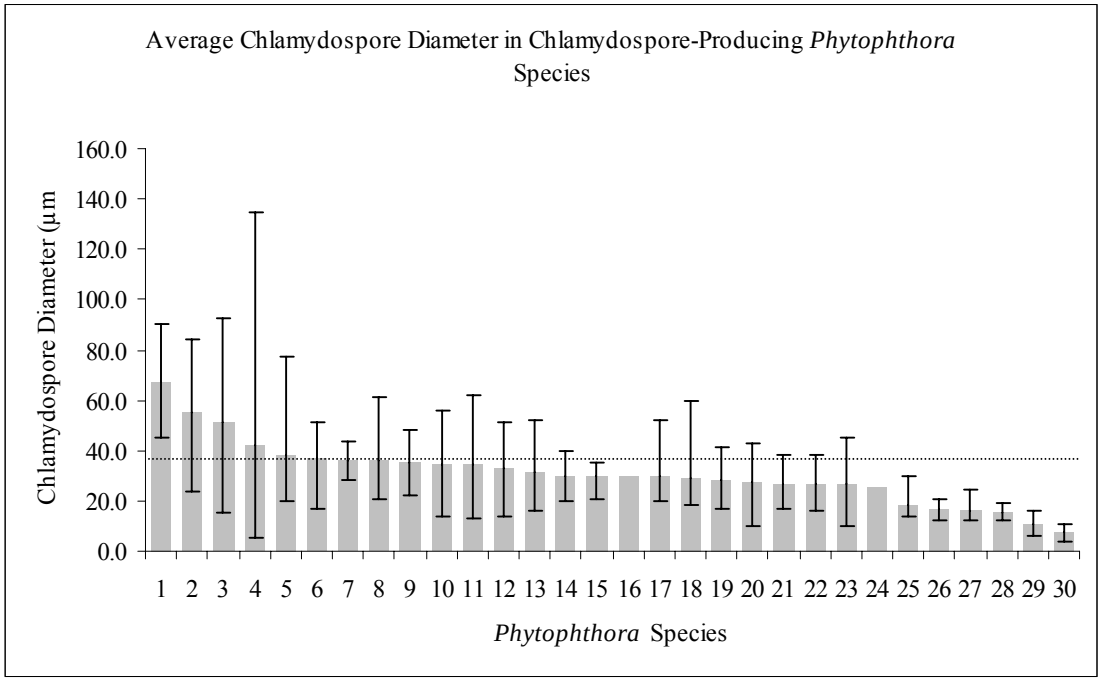
Results

Figure 3: Average Wall Thickness of Chlamydospore-Producing *Phytophthora* Species



Average Chlamydospore Wall Thickness of Chlamydospore-Producing Species: (1) *P. undulata* (2) *P. macrochlamydospora* (3) *P. colocasiae* (4) *P. ramorum* (5) *P. nicotianae (syn. P. parasitica)* (6) *P. capsici* (7) *P. boehmeriae* (8) *P. palmivora* (9) *P. meadii* (10) *P. cactorum* (11) *P. drechsleri* (12) *P. cinnamomi* (13) *P. arecae* (14) *P. citrophthora* (15) *P. insolita* (16) *P. lateralis* (17) *P. megasperma* (18) *P. polygoni* (19) *P. tentaculata.* References for graph in Appendix A. Bars equal range of values. Dotted Horizontal Lines: < 1.0 µm "Thin", 1.0-1.5 µm "Intermediate", and > 1.5 µm "Thick" (Kadooka & Ko 1973).

Figure 4: Average Chlamydospore Diameter in Chlamydospore-Producing *Phytophthora* Species



Average Chlamydospore Diameters of Chlamydospore-Producing Species: (1) *P. quininea* (2) *P. macrochlamydospora* (3) *P. ramorum* (4) *P. cinnamomi* (5) *P. lateralis* (6) *P. boehmeriae* (7) *P. mexicana* (8) *P. undulata* (9) *P. insolita* (10) *P. cactorum* (11) *P. nicotianae (syn. P. parasitica)* (12) *P. palmivora* (13) *P. melonis* (14) *P. megakarya* (15) *P. porri* (16) *P. sojae* (17) *P. capsici* (18) *P. arecae* (19) *P. iranica* (20) *P. citrophthora* (21) *P. colocasiae* (22) *P. meadii* (23) *P. tentaculata* (24) *P. syringae* (25) *P. botryosa* (26) *P. vignae* (27) *P. polygoni* (28) *P. katsurae* (29) *P. megasperma* (30) *P. drechsleri*. References for graph in Appendix A. Bars equal range of values. Dotted Horizontal Line is the Chlamydospores Diameter Cutoff in *Phytophthora*: < 35 µm "Small", > 35 µm "Large" (Erwin & Ribeiro 1996) [qualifiers in quotations added].

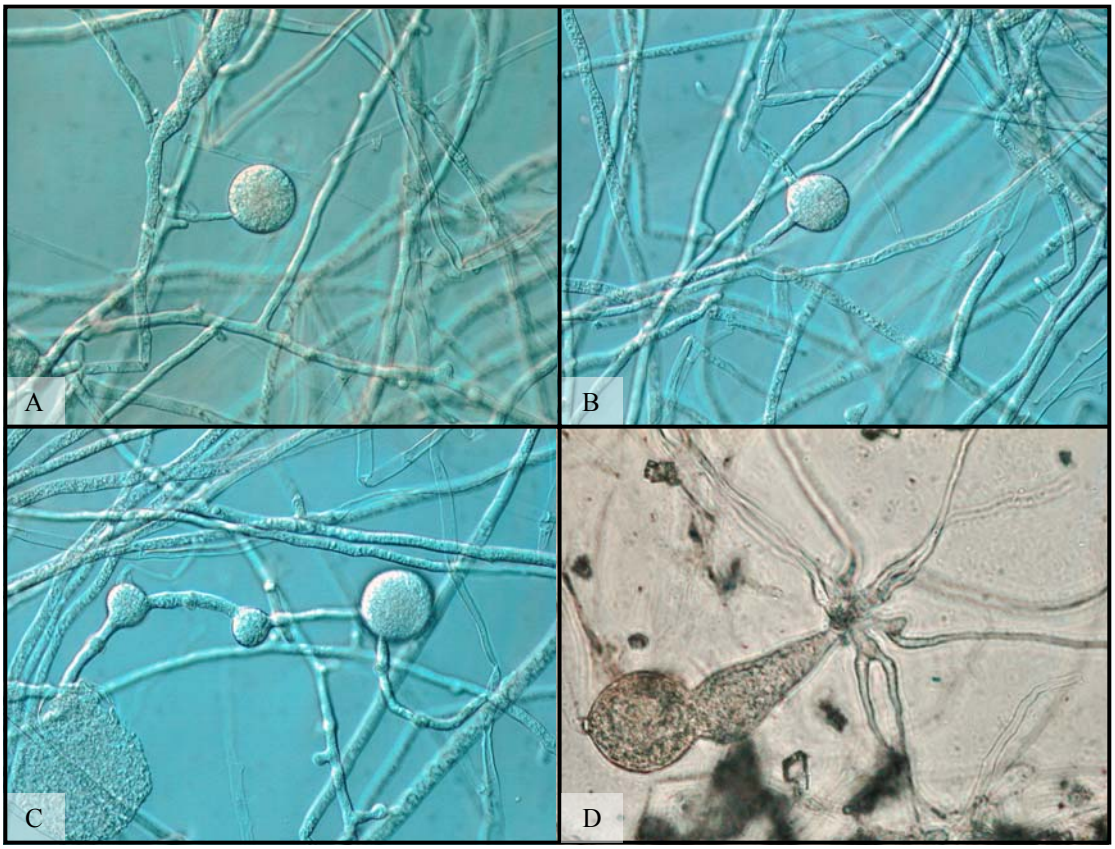
Chlamydospore Formation

Chlamydospores in V8 Agar and V8JB Cultures: Chlamydospores of *P. ramorum* formed in V8 agar and V8JB cultures in large quantities. Preliminary results indicated that chlamydospore formation began within two to three days on V8 agar and V8JB, with cultures containing large numbers of mature and juvenile chlamydospores by seven days on V8 agar and ten days in V8JB. Chlamydospores were formed most commonly in terminal (Figure 5 A) and less frequently in intercalary locations (Figure 5 B & C). Terminal orientations were always characterized by a solitary subtending hypha (by definition) whereas the intercalary configuration was characterized most commonly by two connecting hyphal branches, or more infrequently three hyphal connections. Intercalary chlamydospores were observed to form most often singly (Figure 5 B) and less often in chains (Figure 5 C). Chlamydospores were most commonly spherical, especially when mature. Ovoid

(Figure 5 B) and irregular (Figure 5 D) morphologies were observed to occur most often in chlamydospores at various stages of early development and less frequently at full maturity. Some chlamydospores had a single papilla (a small rounded process) (Figure 7 I, M, N, P-S, & Z) that was more commonly seen in younger chlamydospores.

Chlamydospores at many different stages of development were present in hyphal colonies on V8 agar and V8JB. Older portions of the hyphal colony had a higher proportion of chlamydospores in later stages of development than younger portions of the colony, although chlamydospores were observed to continue to form and develop in older portions of the colony. V8 agar cultures only exhibited radial growth from the point of inoculation. In V8JB, it was frequently observed that more than six hyphal colonies (the number of inoculating LTS plugs) formed after ten days of growth, presumably caused by zoospore release and germination.

Figure 5: *Phytophthora ramorum* Chlamydospore Morphology

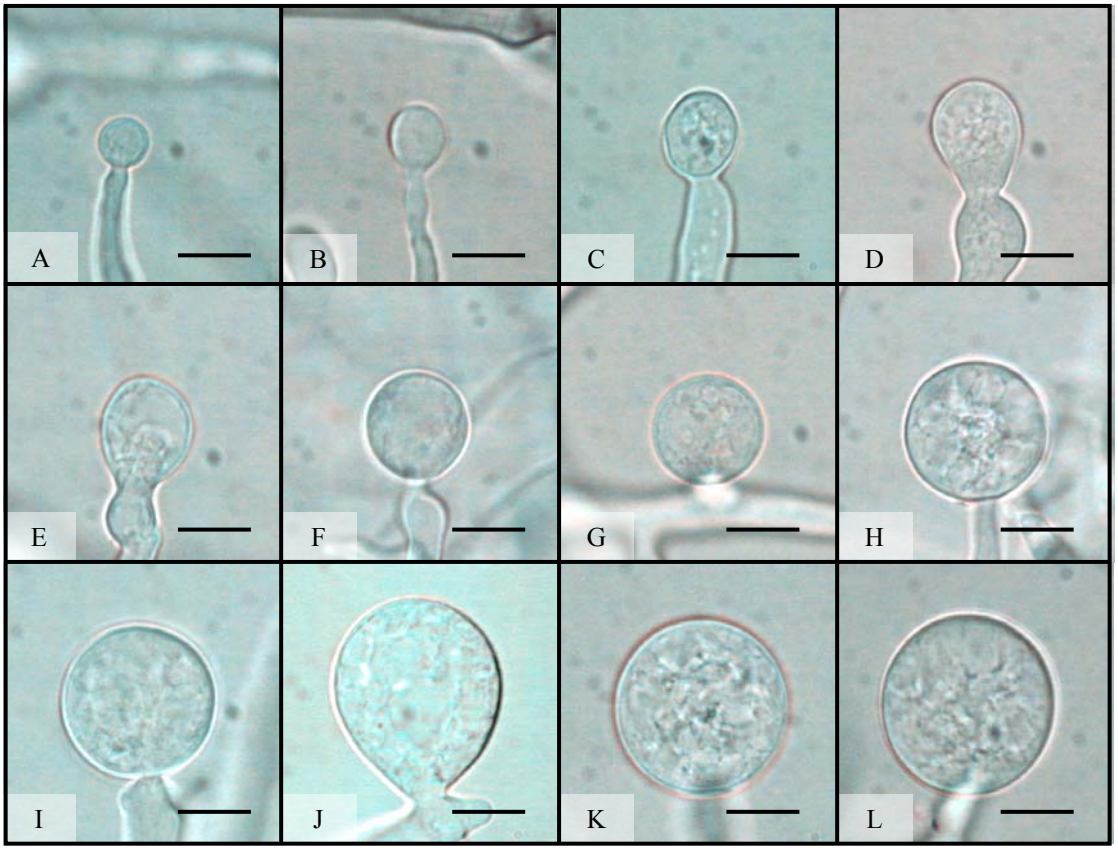


Chlamydospore Morphology: (A) Single terminal chlamydospore (spherical). (B) Single intercalary chlamydospore (ovoid). (C) Chain of intercalary chlamydospores (spherical). (D) Single irregular chlamydospore. A-C: Ten-day-old V8JB-grown chlamydospores of isolate 2027.1 on V8 agar in DIH_2O at 200x magnification under Nomarski illumination. D: Ten-day-old V8JB-grown chlamydospore of isolate P-110 on V8 agar in DIH_2O at 400x magnification under bright field illumination.

Chlamydospore Diameter and Wall Thickness Development: Chlamydospore diameter and wall thickness increased over time. Chlamydospore formation began with the swelling of hyphal walls of the supporting portion of hyphae (Figure 6). Nascent chlamydospore walls were between 0.2 and 0.5 µm thick (Figure 9 A), about the same thickness as the parental hyphal wall (Figure 6 C, E, F). Nascent chlamydospores were filled with cytoplasm that appeared similar to the parental hyphae (Figure 6 D, E, J). Nascent chlamydospores continued to expand until septum formation (Figure 8 A, B, C). Upon formation of the septum, chlamydospore walls began to thicken (Figure 9). Shortly following septation, chlamydospore wall thicknesses were between 0.75 to 1.0 µm (Figure 9 A, B) and increased to a maximum of 5.0 µm in 120 days (Figure 9 I, Table 3) in some chlamydospores. Chlamydospore diameters increased from hyphal swellings 5.0-8.0 µm to 93.0 µm (chlamydospore not shown) (Figures

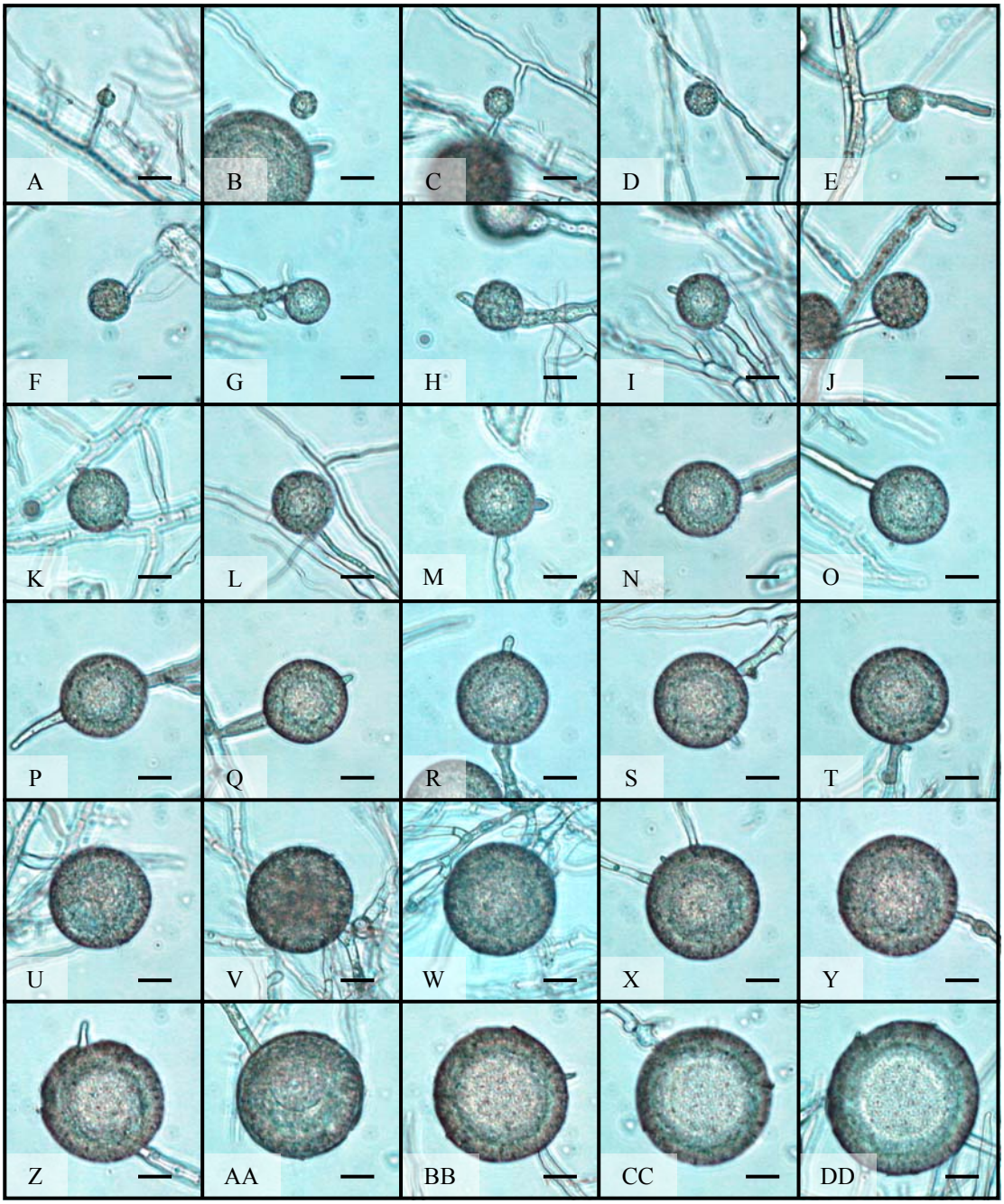
6 & 7). The color of the chlamydospore under bright field illumination changed from hyaline in nascent (prior to septum formation) chlamydospores (Figure 6 & Figure 7 A-E) to opaque over time, with shades of red, brown, and black in maturing chlamydospores (Figure 7 F-DD).

Figure 6: *Phytophthora ramorum* Chlamydospore Diameter Development 6.4-24.0 µm



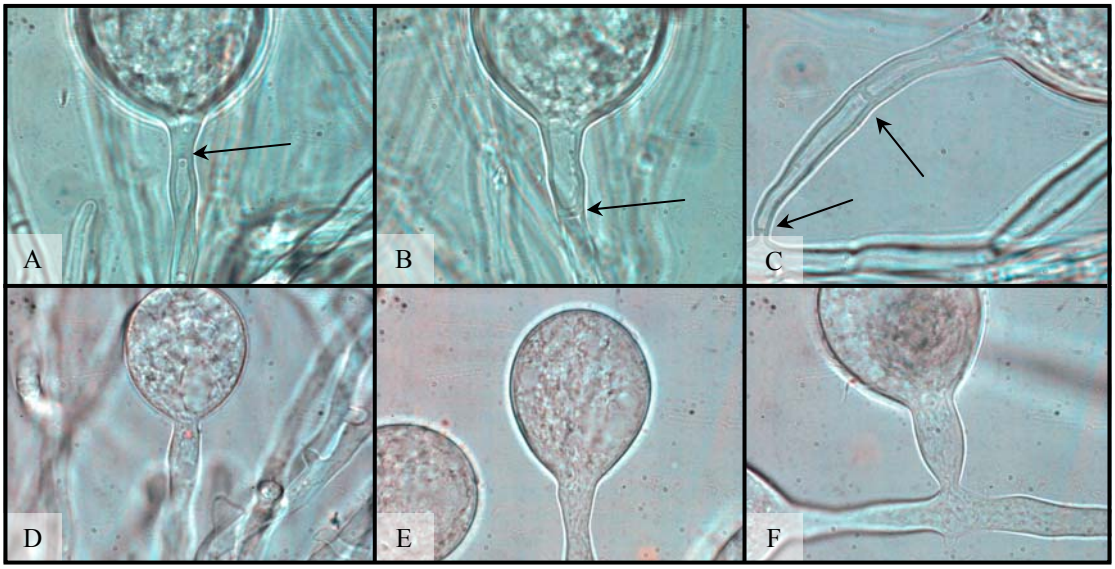
Chlamydospore Diameter Development: (A) 6.4 µm (B) 8.0 µm (C) 10.4 µm (D) 11.2 µm (E) 12.8 µm (F) 14.4 µm (G) 16.0 µm (H) 18.4 µm (I) 19.2 µm (J) 20.8 µm (K) 22.4 µm (L) 24.0 µm. A-L: Eight-day-old V8JB-grown chlamydospores of isolate 2027.1 in lactogycerol at 630x magnification under bright field illumination. Scale bar equals 10 µm.

Figure 7: *Phytophthora ramorum* Chlamydospore Diameter Development 10.3-90.0 µm



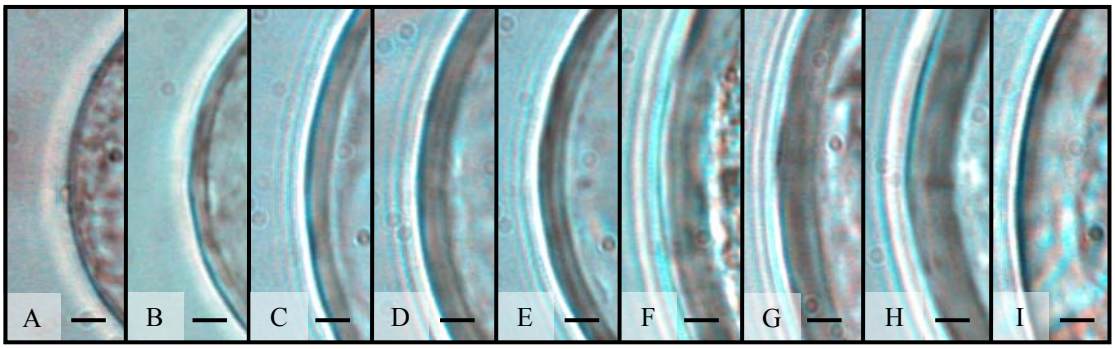
Chlamydospore Diameter Development: (A) 10.3 µm (B) 15.0 µm (C) 17.5 µm (D) 20.0 µm (E) 22.5 µm (F) 25.0 µm (G) 27.5µm (H) 30.0 µm (I) 32.5 µm (J) 35.0 µm (K) 37.5 µm (L) 40.0 µm (M) 42.5 µm (N) 45.0 µm (O) 47.5 µm (P) 50.0 µm (Q) 52.5 µm (R) 55.0 µm (S) 57.5 µm (T) 60.0 µm (U) 62.5 µm (V) 65.0 µm (W) 67.5 µm (X) 70.0 µm (Y) 72.5 µm (Z) 75.0 µm (AA) 77.5 µm (BB) 80.0 µm (CC) 85.0 µm (DD) 90.0 µm. A-DD: Ten- to 120-day-old V8JB-grown chlamydospores of isolate 2027.1 in DIH_2O at 200x magnification under bright field illumination. Scale bar equals 20 µm.

Figure 8: *Phytophthora ramorum* Chlamydospore Septum Development



Chlamydospore Septum Development: (A-C) Chlamydospores with fully developed septa (arrow). (D-F) Nascent chlamydospores without septa. A-C and D-F: 48- and 8-day-old (respectively) V8JB-grown chlamydospores of isolate 2027.1 in lactoglycerol at 1000x under bright field illumination.

Figure 9: *Phytophthora ramorum* Chlamydospore Wall Thickness Development



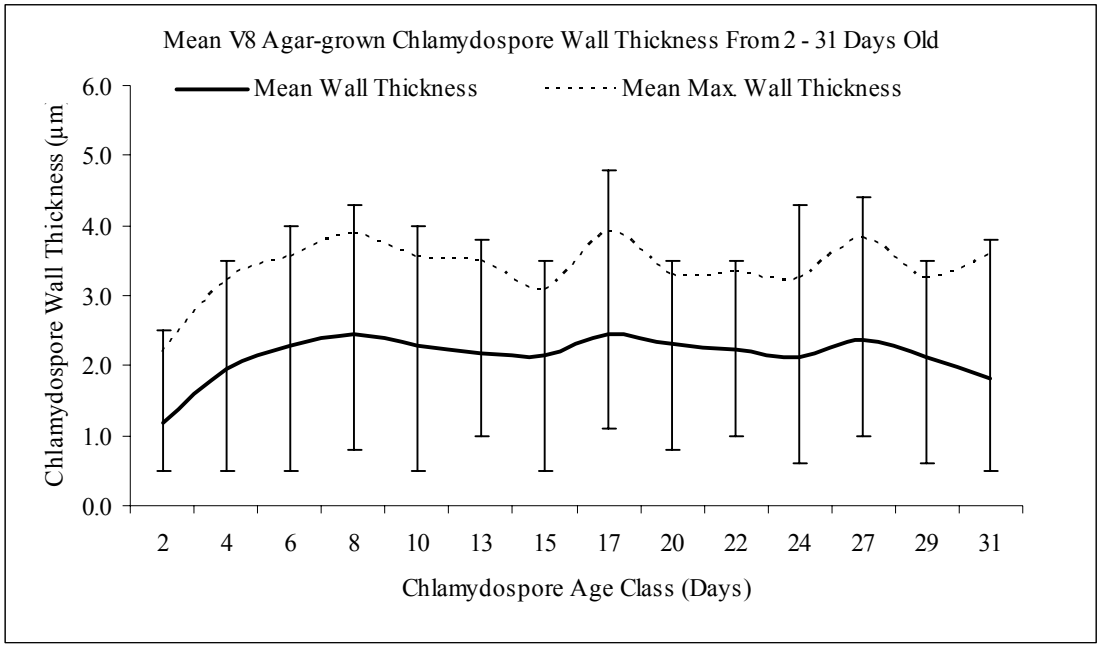
Chlamydospore Wall Thickness Development: (A) 0.5 µm (B) 1.0 µm (C) 1.5 µm (D) 2.0 µm (E) 2.5 µm (F) 3.0 µm (G) 3.5 µm (H) 4.0 µm (I) 5.0 µm. V8JB-grown chlamydospores of isolate 2027.1 grown for: (A) 7, (B) 11, (C, F, G, and I) 223, and (D, E, and H) 425 days. Depicted in lactoglycerol at 1000x magnification under brightfield illumination. Scale bar equals 2 µm.

Chlamydospore Maturation on V8 Agar: On V8 agar, mean chlamydospore wall thickness (MCWT) increased from 1.2 µm in the two-day-old age class to 2.5 µm in the eight day age class; wall thickness averaged 2.2 µm in chlamydospore age classes ten to 31 days (Figure 10, Table 1). This pattern was repeated among the chlamydospores with the thickest walls (and thus potentially the oldest) in each age class. Mean maximum chlamydospore wall thickness values increased from 2.2 µm in the

two day age class to 3.9 µm in the eight day age class and then averaged 3.5 µm in chlamydospores in the ten to 31-day-old age classes (Figure 10, Table 1).

The percentage of chlamydospores having wall thicknesses 0.5 to 1.4 µm decreased from 75 % in two-day-old age class chlamydospores to an average of 8 % in chlamydospores from the 8 to 31 day age classes (Figure 11). Chlamydospores with wall thicknesses 1.5 to 1.9 µm were evenly represented at an average of 20 % in chlamydospores in the two- to 31-day-old age classes (Figure 11). Chlamydospores with wall thicknesses 2.0 to 2.9 µm increased from 7 % in two day age class chlamydospores to an average of 59 % in chlamydospores in the four- to 31-day-old age classes (Figure 11). Chlamydospores with wall thicknesses 3.0 to 3.9 µm averaged 12 % of chlamydospores in the four- to 31-day-old age classes (Figure 11). The percentage of chlamydospores with wall thicknesses 4.0 to 4.4 µm averaged 0.3 % of chlamydospores in the 6- to 31-day-old age classes (Figure 11). Only one chlamydospore had a wall thickness of 4.75 µm at 17 days of age (Figure 10, Table 1).

Figure 10: Mean V8 Agar-grown Chlamydospore Wall Thickness From 2-31 Days Old

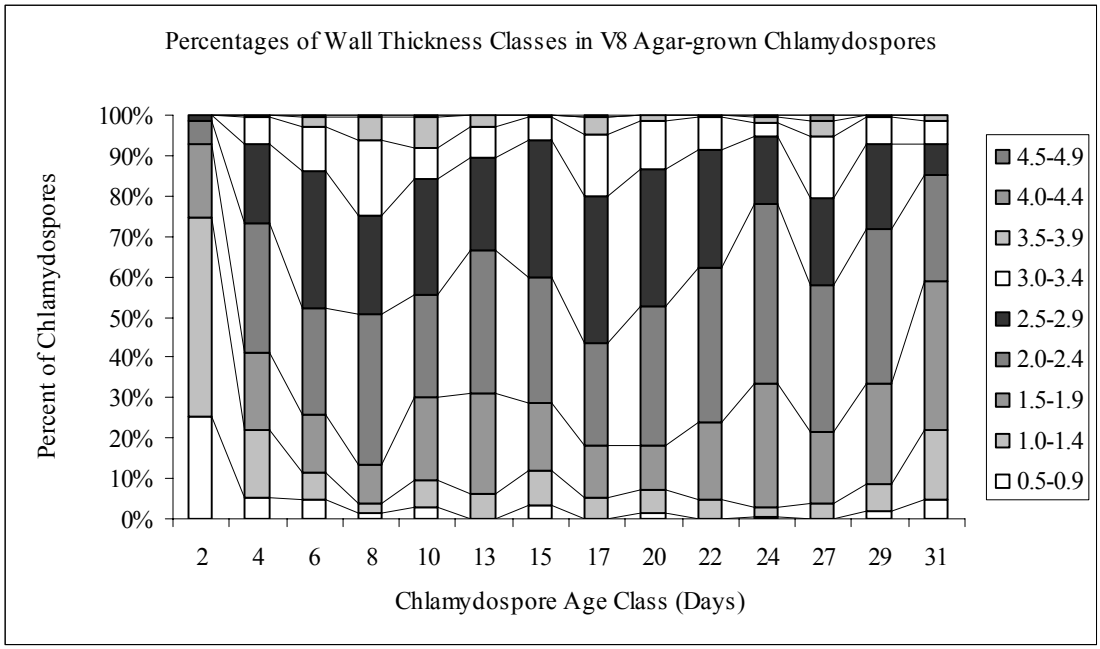


Bars equal the range of chlamydospore wall thickness values in each age class.

Table 1: Mean Chlamydospore Wall Thickness (MCWT) on V8 Agar

Chlamydospore Age Class (Days)	All Replications MCWT (Range) (μm)	Replication 1 MCWT (Range) (μm)	Replication 2 MCWT (Range) (μm)	Replication 3 MCWT (Range) (μm)
2	1.2 (0.5-2.5)	1.2 (0.6-2.5)	1.0 (0.5-1.6)	1.4 (0.5-2.5)
4	2.0 (0.5-3.5)	1.9 (0.5-3.1)	2.0 (0.5-3.5)	2.0 (0.9-3.0)
6	2.3 (0.5-4.0)	2.2 (0.6-3.5)	2.3 (0.5-3.1)	2.3 (1.0-4.0)
8	2.5 (0.8-4.3)	2.5 (0.8-3.9)	2.5 (1.8-3.5)	2.5 (0.8-4.3)
10	2.3 (0.5-4.0)	1.7 (0.5-2.9)	2.5 (1.3-4.0)	2.6 (0.8-3.8)
13	2.2 (1.0-3.8)	2.0 (1.0-3.8)	2.3 (1.3-3.5)	2.3 (1.3-3.3)
15	2.1 (0.5-3.5)	2.2 (0.8-3.5)	1.9 (0.8-2.8)	2.3 (0.5-3.0)
17	2.4 (1.1-4.8)	2.5 (1.5-4.8)	2.4 (1.1-3.5)	2.4 (1.3-3.5)
20	2.3 (0.8-3.5)	2.1 (0.8-2.9)	2.3 (1.0-3.5)	2.5 (1.0-3.5)
22	2.2 (1.0-3.5)	2.0 (1.0-3.5)	2.3 (1.4-3.3)	2.4 (1.3-3.3)
24	2.1 (0.6-4.3)	2.0 (1.5-2.5)	2.1 (1.3-3.0)	2.3 (0.6-4.3)
27	2.4 (1.0-4.4)	2.3 (1.0-4.4)	2.3 (1.3-3.4)	2.5 (1.5-3.8)
29	2.1 (0.6-3.5)	2.0 (1.0-3.5)	2.1 (0.6-3.0)	2.2 (0.9-3.3)
31	1.8 (0.5-3.8)	1.7 (0.5-3.3)	1.8 (0.8-3.8)	1.9 (0.6-3.8)

Figure 11: Percentages of Wall Thickness Classes in V8 Agar-grown Chlamydospores

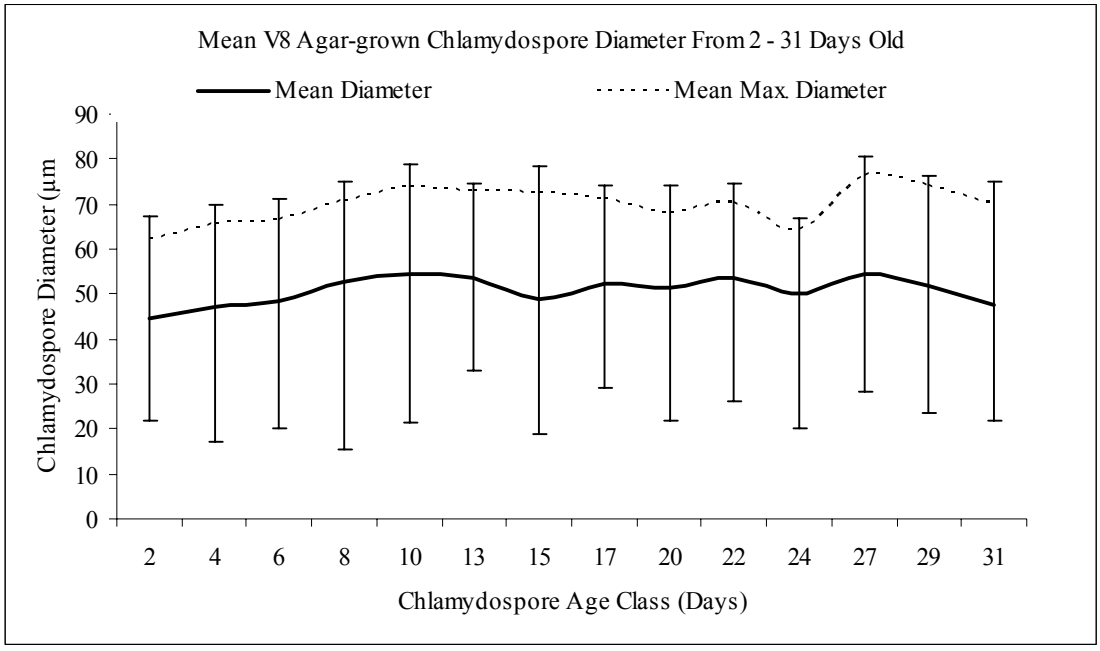


Mean chlamydospore diameter (MCD) increased from 44.7 μm in two-day-old age class chlamydospores to 54.3 μm in the ten-day-old age class and then averaged 51.5 μm in the 13 to 31 day old age class chlamydospores (Figure 12, Table 2). This pattern was repeated in mean maximum chlamydospore diameter values that increased from 62.1 μm in the two day age class to 73.8 μm in the

ten day age class and then averaged 71.1 µm in chlamydospores in the 13- to 31-day-old age classes (Figure 12, Table 2).

The percentage of chlamydospores with diameters 10.0 to 39.9 µm decreased from 27 % in two-day-old age class chlamydospores to an average of 9 % in chlamydospores in the ten to 31 day age classes (Figure 13). Chlamydospores with diameters 40.0 to 59.9 µm were evenly represented at an average of 70 % in chlamydospores in the two to 31 day old age classes (Figure 13). Chlamydospores with diameters 60.0 to 69.9 µm increased from 3 % in two-day-old age class chlamydospores to an average of 17 % in chlamydospores in the ten- to 31-day-old age classes (Figure 13). Chlamydospores with diameters 70.0 to 79.9 µm increased from 0.7 % in four-day-old age class chlamydospores to an average of 4 % in chlamydospores in the 10- to 31-day-old age classes (Figure 13). Only one chlamydospore had a diameter of 80.5 µm at 27 days old (Figure 12, Table 2).

Figure 12: Mean V8 Agar-grown Chlamydospore Diameter From 2-31 Days Old

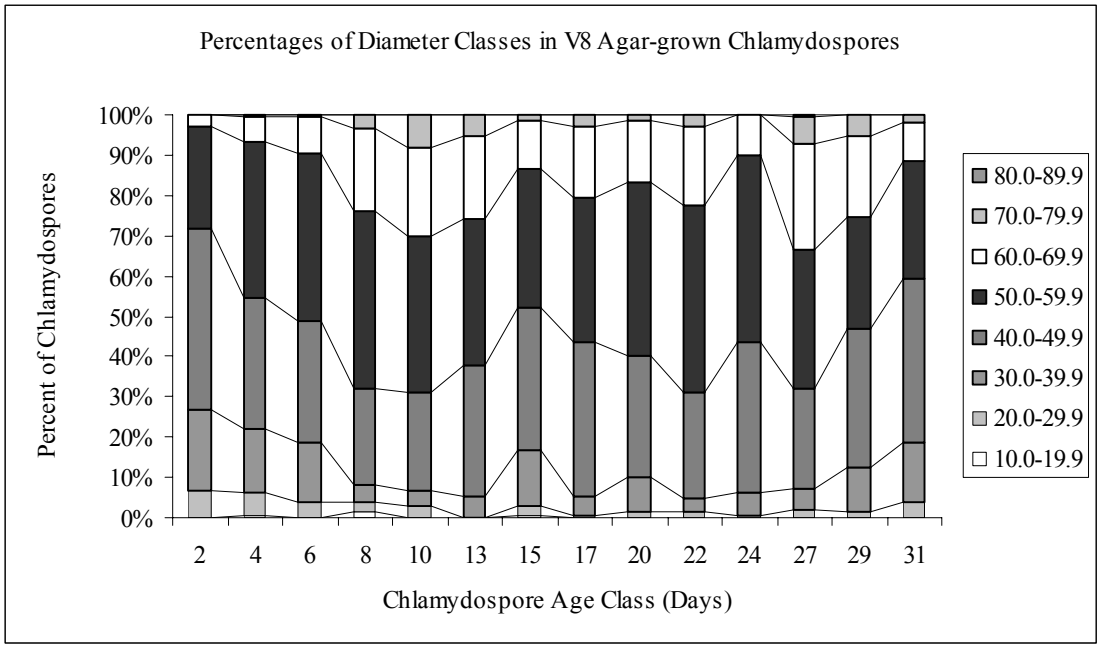


Bars equal the range of chlamydospore diameter values in each age class.

Table 2: Mean Chlamydospore Diameter (MCD) on V8 Agar

Chlamydospore Age Class (Days)	All Replications MCD (Range) (μm)	Replication 1 MCD (Range) (μm)	Replication 2 MCD (Range) (μm)	Replication 3 MCD (Range) (μm)
2	44.7 (22.0-67.5)	47.7 (22.0-67.5)	42.5 (22.0-63.0)	43.8 (31.0-55.8)
4	47.1 (17.0-70.0)	43.5 (17.0-58.0)	53.1 (27.0-69.0)	44.8 (22.0-70.0)
6	48.4 (20.3-71.0)	46.0 (20.3-60.5)	47.7 (27.0-67.5)	51.6 (26.5-71.0)
8	52.8 (15.5-75.0)	49.1 (19.0-68.0)	54.3 (34.5-69.0)	55.0 (15.5-75.0)
10	54.3 (21.5-79.0)	46.7 (21.5-67.5)	60.4 (43.0-79.0)	55.8 (37.0-75.0)
13	53.6 (33.0-74.5)	51.6 (35.0-73.5)	51.6 (38.0-71.0)	57.5 (33.0-74.5)
15	48.9 (19.0-78.5)	46.2 (28.5-64.0)	48.7 (25.5-75.0)	51.8 (19.0-78.5)
17	52.3 (29.0-74.0)	52.5 (33.0-67.8)	54.3 (30.0-72.0)	50.0 (29.0-74.0)
20	51.5 (22.0-74.0)	51.6 (27.5-68.0)	53.4 (32.5-74.0)	49.4 (22.0-63.0)
22	53.4 (26.0-74.5)	56.7 (26.0-74.0)	54.4 (34.5-74.5)	49.0 (27.0-62.5)
24	50.1 (20.0-67.0)	49.0 (39.3-62.0)	49.9 (33.5-64.5)	51.5 (20.0-67.0)
27	54.5 (28.4-80.5)	53.2 (28.5-73.0)	55.3 (28.5-80.5)	55.0 (35.5-75.0)
29	51.9 (23.5-76.5)	50.4 (32.5-75.5)	50.6 (23.5-76.5)	54.5 (36.5-70.0)
31	47.4 (22.0-75.0)	46.3 (22.0-65.0)	48.4 (23.0-69.5)	47.5 (28.5-75.0)

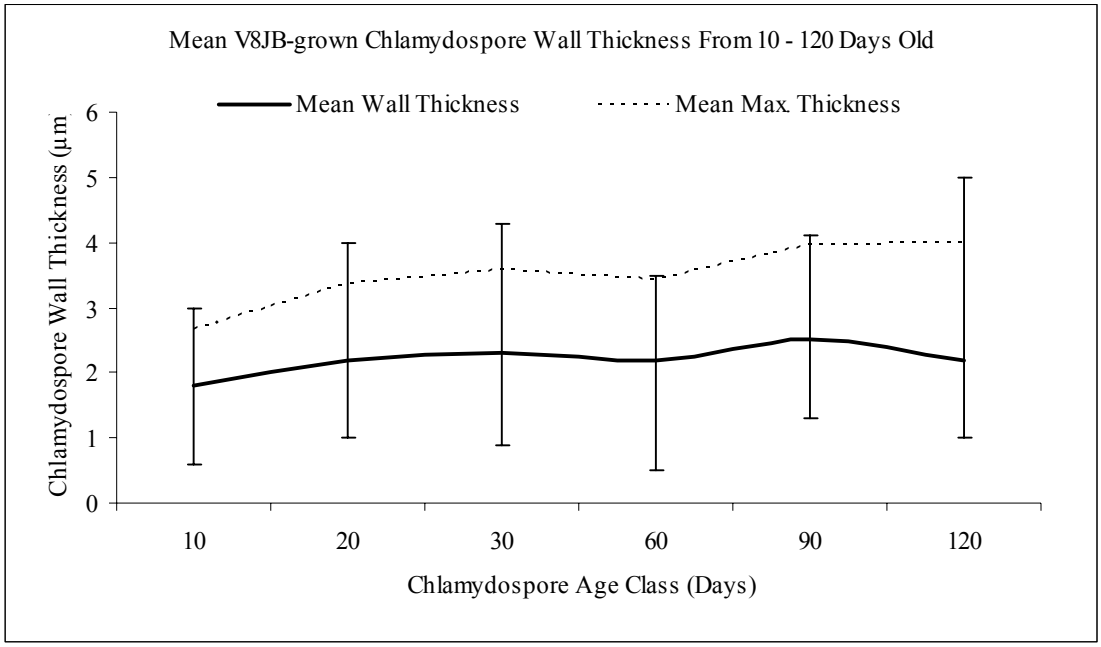
Figure 13: Percentages of Diameter Classes in V8 Agar-grown Chlamydospores



Chlamydospore Maturation in V8JB: Mean chlamydospore wall thickness (MCWT) increased from 1.8 μm in ten-day-old age class chlamydospores to an average of 2.3 μm in chlamydospores in the 20 to 120 day old age classes (Figure 14, Table 3). Mean maximum chlamydospore wall thickness continued to increase from 2.7 μm in the ten-day-old age class to 4.0 μm in the 90- and 120-day-old age classes (Figure 14).

The percentage of chlamydospores with wall thicknesses 0.5 to 1.9 µm decreased from 60 % in ten-day-old age class chlamydospores to an average of 23 % in 90- and 120-day-old age class chlamydospores (Figure 15). Chlamydospores with wall thicknesses 2.0 to 2.4 µm were evenly represented at an average of 33 % in chlamydospores in the ten- to 120-day-old age classes (Figure 15). Chlamydospores with wall thicknesses 2.5 to 2.9 µm increased from 4 % in ten-day-old age class chlamydospores to an average of 27 % in chlamydospores in the 20- to 120-day-old age classes (Figure 15). Chlamydospores with wall thicknesses 3.0 to 3.4 µm increased from 0.7 % in ten-day-old age class chlamydospores to and average of 10 % in chlamydospores in the 20- to 120-day-old age classes (Figure 15). Chlamydospores with wall thicknesses 3.5 to 4.4 µm were evenly represented at an average of 4 % in chlamydospores in the 20- to 120-day-old age classes (Figure 15). Only one chlamydospore had a wall thickness of 5.0 µm at 120 days of age (Figure 14, Table 3).

Figure 14: Mean V8JB-grown Chlamydospore Wall Thickness From 10-120 Days Old

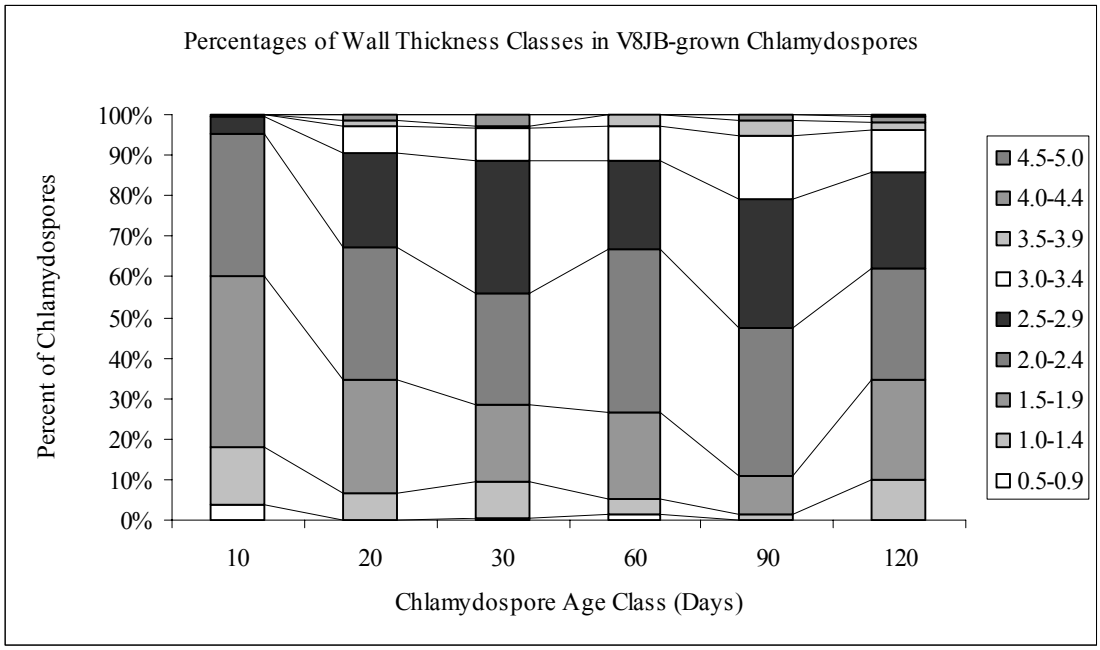


Bars equal the range of chlamydospore wall thickness values in each age class.

Table 3: Mean Chlamydospore Wall Thickness (MCWT) in V8JB

Chlamydospore Age Class (Days)	All Replications MCWT (Range) (µm)	Replication 1 MCWT (Range) (µm)	Replication 2 MCWT (Range) (µm)	Replication 3 MCWT (Range) (µm)
10	1.8 (0.6-3.0)	1.7 (0.6-2.5)	1.8 (0.9-3.0)	1.8 (0.8-2.5)
20	2.2 (1.0-4.0)	1.8 (1.3-2.9)	2.1 (1.0-3.3)	2.6 (1.4-4.0)
30	2.3 (0.9-4.3)	2.0 (0.9-3.0)	2.2 (1.0-3.5)	2.5 (1.0-4.3)
60	2.2 (0.5-3.5)	2.1 (0.5-3.5)	2.2 (1.1-3.5)	2.3 (1.5-3.3)
90	2.5 (1.3-4.1)	2.6 (1.8-4.1)	2.4 (1.3-3.8)	2.5 (1.5-4.0)
120	2.2 (1.0-5.0)	1.8 (1.1-2.8)	2.3 (1.0-5.0)	2.5 (1.0-4.3)

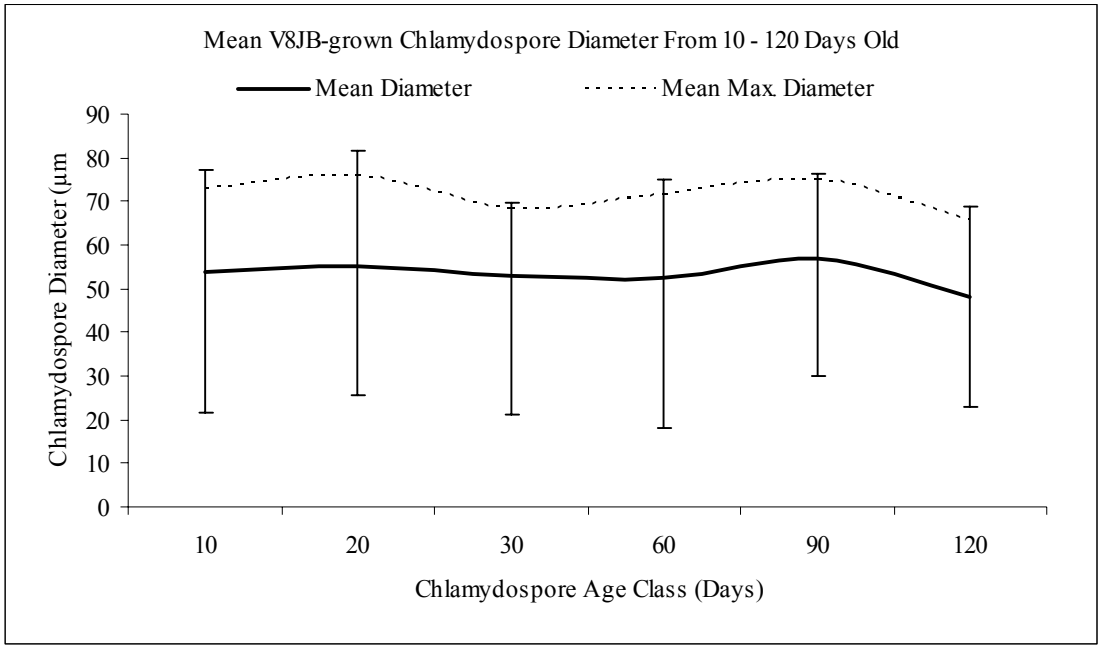
Figure 15: Percentages of Wall Thickness Classes in V8JB-grown Chlamydospores



Mean diameter (MCD) of chlamydospores formed in V8JB averaged 53.2 µm in ten to 120 day age classes (Figure 16, Table 4). This pattern was repeated in mean maximum chlamydospore diameter values that averaged 71.6 µm in chlamydospores in the ten- to 120-day-old age classes (Figure 16).

The percent of chlamydospores with diameters 10.0 to 39.9 µm, 40.0 to 59.9 µm, and 60.0 to 69.9 µm were evenly represented at averages of 8 %, 68 %, and 20 % respectively in chlamydospores in the ten- to 120-day-old age classes (Figure 17). Chlamydospores with diameters 70.0 to 79.9 µm averaged 4 % in chlamydospores in the ten- to 90-day-old age classes, but were not represented in the 30- or 120-day-old age classes (Figure 17). Only two chlamydospores had a diameter of greater than 80.0 µm at 20 days of age (Figure 16, Table 4).

Figure 16: Mean V8JB-grown Chlamydospore Diameter From 10-120 Days Old

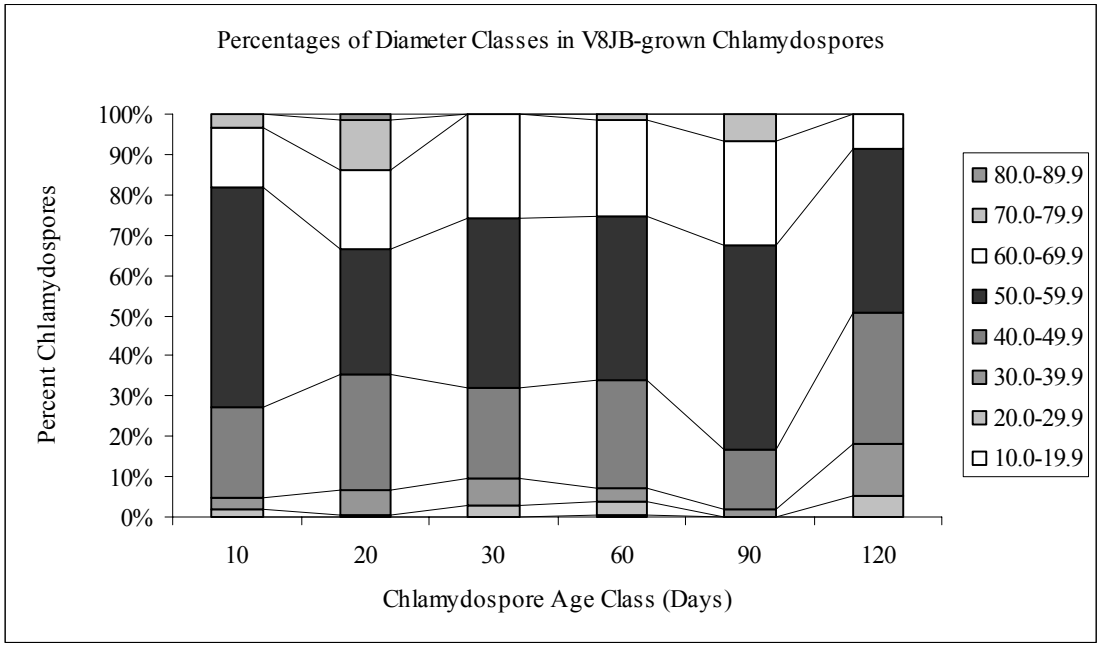


Bars equal the range of chlamydospore diameter values in each age class.

Table 4: Mean Chlamydospore Diameter (MCD) in V8JB

Chlamydospore Age Class (Days)	All Replications MCD (Range) (µm)	Replication 1 MCD (Range) (µm)	Replication 2 MCD (Range) (µm)	Replication 3 MCD (Range) (µm)
10	53.7 (21.8-77.0)	54.9 (29.0-77.0)	52.8 (21.8-71.0)	53.5 (36.0-71.0)
20	55.3 (25.8-81.8)	48.8 (37.5-73.5)	52.9 (38.0-72.5)	64.3 (25.8-81.8)
30	52.9 (21.0-69.5)	49.1 (27.0-67.0)	55.3 (21.0-69.5)	54.1 (23.8-69.0)
60	52.6 (18.0-75.0)	51.1 (18.0-69.3)	52.0 (24.5-70.0)	54.6 (27.0-75.0)
90	56.8 (30.0-76.5)	54.5 (33.0-72.0)	57.2 (40.0-76.3)	58.6 (30.0-76.5)
120	48.1 (23.0-68.8)	45.8 (27.3-60.8)	47.7 (23.0-68.8)	50.7 (23.0-68.0)

Figure 17: Percentages of Diameter Classes in V8JB-grown Chlamydospores



Relationship Between Chlamydospore Diameter and Wall Thickness: Chlamydospore diameter increased with wall thickness in chlamydospores grown on V8 agar for two to 31 days (Figure 18) and in chlamydospores grown in V8JB for ten to 120 days (Figure 19). The r^2 values for V8 agar and V8JB-grown chlamydospores were 0.47 (Figure 18) and 0.37 respectively (Figure 19).

Figure 18: Relationship Between Diameter and Wall Thickness in V8 Agar-grown Chlamydospores

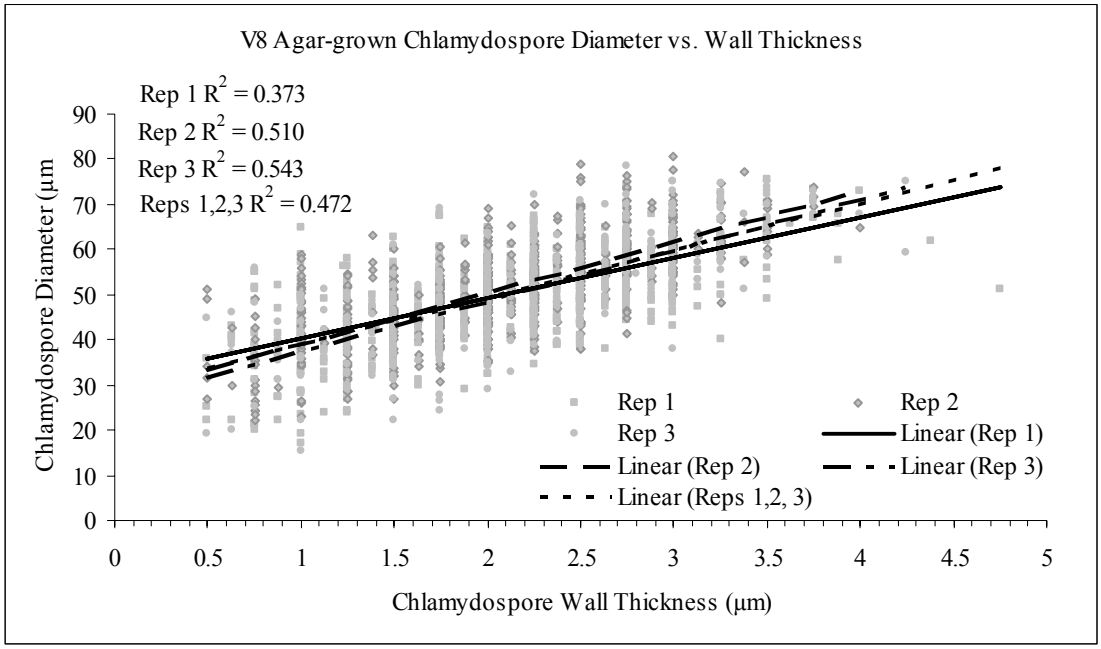
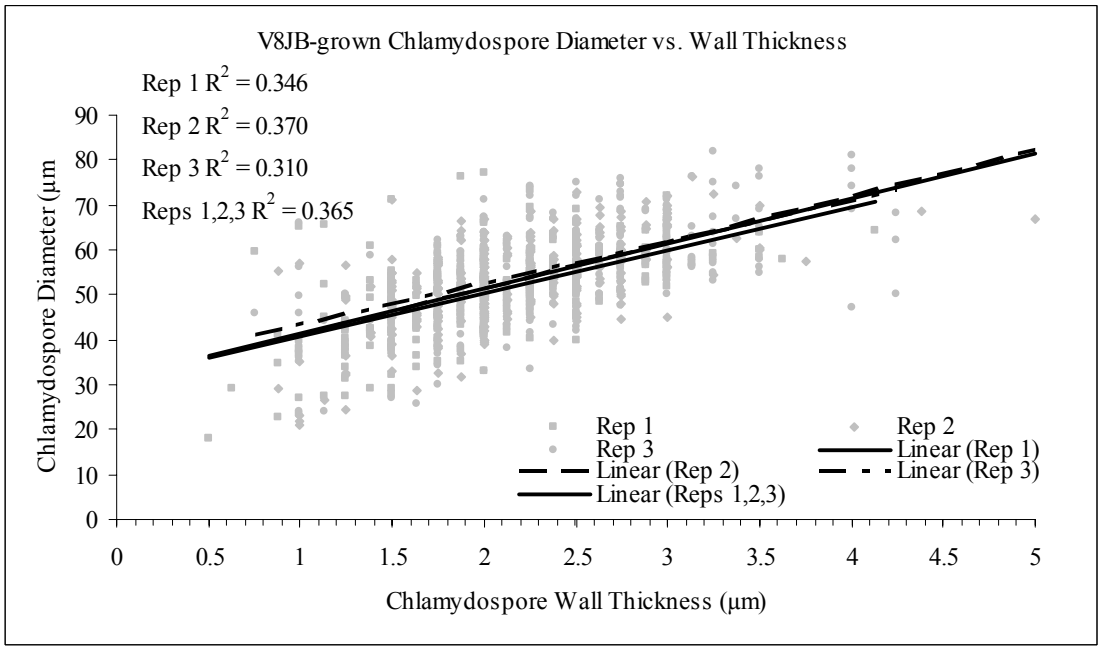


Figure 19: Relationship Between Diameter and Wall Thickness in V8JB-grown Chlamydospores



Discussion

Compared with at least 19 other species of *Phytophthora, P. ramorum* chlamydospore walls are the fourth thickest and have the third largest average diameter. *P. ramorum* produces its chlamydospores abundantly in V8 agar and V8JB cultures at 20 C in the dark starting within two to three days of inoculation. By eight days the average chlamydospore wall has thickened from between 0.2 and 0.5 µm to 2.3 +/- 0.1 µm and by ten days has fully expanded from a hyphal swelling to an average diameter of 52.4 +/- 0.9 µm. Further enlargement of wall thickness and diameter then ceases for most chlamydospores, but walls may continue to enlarge to a thickness of 5.0 µm, and expand to diameters as great as 93.0 µm. Cultures of *P. ramorum* may contain chlamydospores in various stages of development, but after ten to twenty days the frequency distribution of ranges of wall thicknesses and diameters remains relatively constant in cultures at least up to 120 days. There is a strong relationship between chlamydospore wall thickness and diameter; chlamydospore wall thickness tends to increase with chlamydospore diameter.

There are at least 35 species of *Phytophthora* reported to produce chlamydospores to at least some degree. The range of *Phytophthora* chlamydospore wall thicknesses and diameters reported in the literature includes, 19 species for which some information is available on chlamydospore wall thickness and 30 species for which information on chlamydospore diameters exists. Both characteristics are used to some extent to aid in the identification of species, but chlamydospore diameter is more often cited and more often used for the identification. In cases where chlamydospore wall thickness was cited, it was usually described as "thin" or "thick" rather than a measured value. Consequently, the importance of chlamydospore wall thickness has remained relatively poorly defined within the genus. Often these descriptors such as "thin" and "thick" are used in reference to a particular species and are only informative in the context of the research of that particular species, not the full range of wall thickness values that are found in the genus. When discussing "thin" and "thick" walled chlamydospores for research on chlamydospores of *P. cinnamomi,* "thin" is defined as < 1.0 µm and "thick" is defined as ≥ 1.0 µm. This arbitrary definition makes intuitive sense when the relatively thin-walled chlamydospores of *P. cinnamomi* (Figure 3, 12) are considered without reference to other species within the genus. This definition does not make sense, however, for *P. ramorum*, which has nascent chlamydospores with walls as thick as those of fully developed chlamydospores of *P. cinnamomi*. In *P. ramorum*, the average chlamydospore wall is only slightly thinner than the thickest chlamydospore wall reported for *P. cinnamomi*. Applying a similar arbitrary definition of thin- and thick-walled chlamydospores for *P. ramorum* alone, a thin wall would be < 1.5 µm and a thick wall would be > 2.5 µm. Such a definition is not proposed here, as this would only add to the confusion of what is meant when a chlamydospore in *Phytophthora* is referred to only as "thin-" or "thick-" walled.

A more precise working definition of the meaning of thin- and thick-walled chlamydospores is now possible in *Phytophthora*. Of the 19 species with published chlamydospore wall thicknesses reported in the literature (Peterson 1910; Van der Plaats-Niterink 1981; Erwin & Ribeiro 1996; Erwin & Ribeiro 1996; Smith 2007, *unpublished*; Werres *et al.* 2001; Tsao 1991; Uchida & Aragaki 1985; Islam *et al.* 2005; Erwin & Ribeiro 1996; Kadooka & Ko 1973; Dantanarayana *et al*. 1984; Hemmes & Lerma 1985; Dantanarayana *et al*. 1984; Erwin & Ribeiro 1996; Cother & Griffin 1973; Cother & Griffin 1974; Hemmes & Wong 1975; Cother & Griffin 1973; Shew & Benson 1982; Erwin & Ribeiro 1996; Erwin & Ribeiro 1996; Ann & Ko 1980; Tucker & Milbrath 1942; Basu 1980; Zheng & Ho 2000; Erwin & Ribeiro 1996), ten were < 1.5 µm and nine were > 1.5 µm (Figure 3), suggesting 1.5 µm is a useful median value. While working with thin- and thick-walled chlamydospores of *P. palmivora*, Kadooka & Ko (1973) defined thin as < 1.0 µm, added an intermediate value of 1.0 to 1.5 µm, and thick as > 1.5 µm. *P. palmivora* produces a thick-walled chlamydospore by this definition, but the range of variation in this species is greater. It also readily produces chlamydospores that are thinner and thicker over a relatively broad range of values (Figure 3). This definition more closely approximates an equal division of wall thicknesses across *Phytophthora*, rather than describing wall thickness based on relative thickness of each individual species. Although this definition adequately describes the intermediate and thinner-walled species, it is not adequate to describe the thicker-walled species like *P. undulata*, *P. macrochlamydospora*, *P. colocasiae*, and *P. ramorum*. Such difficulties can be mitigated by applying the definition of wall thickness proposed by Kadooka & Ko (1973), which best reflects values across the genus, and then reporting the measured values in the thicker-walled species studied.

P. ramorum produces abundant chlamydospores in V8 agar and V8JB cultures starting within two to three days. There has been no isolate variation recorded related to abundance of chlamydospore production. *Phytophthora* species that produce chlamydospores abundantly do so in a matter of days. *P. cinnamomi* will produce chlamydospores in 24-27 hours (Cahill *et al.* 1989). *P. palmivora* produces chlamydospores within 5 days (Hemmes & Lerma 1985). *P. quininea* produces chlamydospores within 5.5 days (Erwin & Ribeiro 1996). *P. polygoni* produces chlamydospores within 12-15 days (Zheng & Ho 2000). From information available, species that produce chlamydospores infrequently do so over much longer time periods. *P. citricola* produces chlamydospores in 3 weeks (Mchau & Coffey 1994). *P. infestans* has only one report of chlamydospores being produced, which required 4-9 months of incubation (Patrikeyeva 1979).

Chlamydospore walls undergo a maturation process where, at the formation of the septum, a secondary wall is deposited. In *P. ramorum* this process begins as early as four days after inoculation in V8 agar culture in some chlamydospores (Figure 10). In most cultures grown on V8 agar the chlamydospore wall thickens quickly in the first eight days and then ceases to thicken up to 31 days of age (Figure 10). This process is further elucidated in frequency distribution data, which shows the

higher proportions of thinner-walled chlamydospores at two days (93 % 0.5-1.9 µm) decrease to a culture composition of around 30 % between eight and 31 days of age (Figure 11). In some V8 agar-grown chlamydospores, the walls become thicker. In these chlamydospores the walls become much thicker than average, but they exhibit the same trend of ceasing to thicken after eight days (Figure 10).

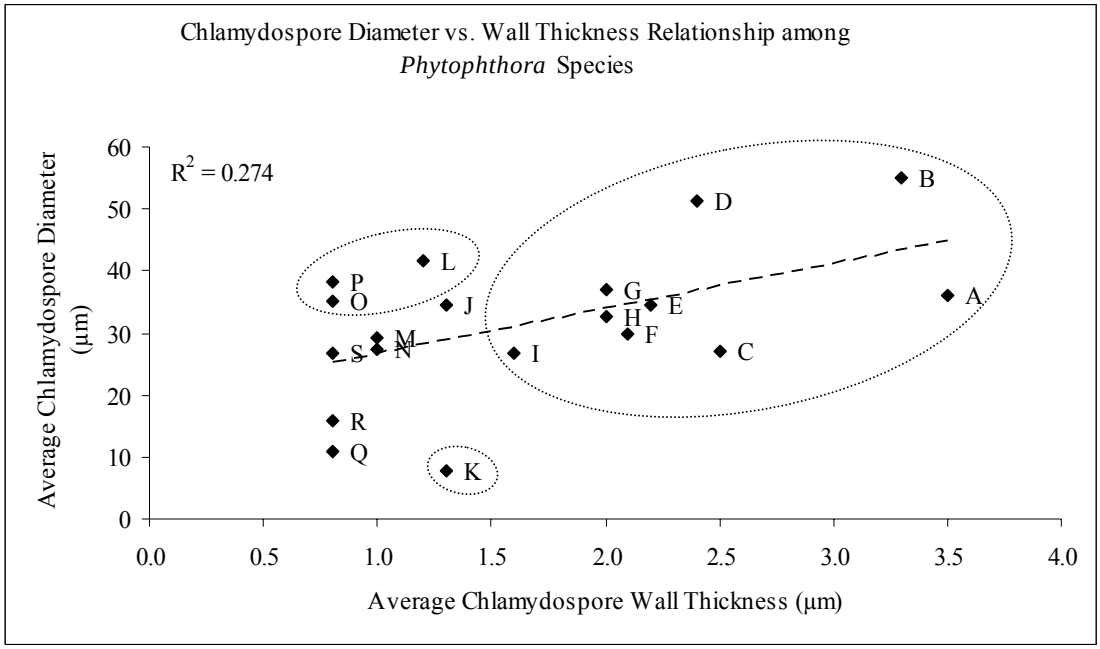
V8JB-grown chlamydospore walls tended to be relatively thinner at 10 days of age (average 1.8 µm) compared to those in V8 agar-grown cultures (average 2.3 µm). In V8JB-grown cultures, chlamydospore walls thickened from 1.8 µm at ten days to an average of 2.3 µm from 20 days to 120 days of age (Figure 14). Also, the proportion of thinner-walled chlamydospores continued to decrease from 60 % 0.5-1.9 µm at the ten days to 35 % at 20 days (Figure 15). Furthermore, mean maximum values average 3.5 µm after eight days in V8 agar (Figure 10), however, V8JB-grown chlamydospore walls tended to continue to enlarge from 2.7 µm at 10 days to 4.0 µm at 90 and 120 days (Figure 14). This is good evidence that chlamydospore wall thickening continues to occur at least up to 120 days of age in *P. ramorum*. Although the 10 day average is different for the two media, the final average is the same, suggesting that the average chlamydospore wall thickening takes place about twice as fast in V8 agar (10 days compared to 20 days).

In V8 agar-grown cultures, the enlargement of chlamydospores exhibits a similar pattern to wall development. Chlamydospores get progressively larger to an average diameter of 51.5 µm at ten days and then cease to expand up to 31 days of age (Figure 12). This pattern is repeated in the most quickly expanding chlamydospores, and therefore the largest, with maximum diameters being attained at between ten to 31 days (Figure 12). In V8JB-grown chlamydospores 10 to 120 days of age, chlamydospore diameters did not increase, but were slightly larger than V8 agar-grown chlamydospores averaging 53.2 µm (Figure 16). This is good evidence that chlamydospore expansion ceases after 10 days of age, suggesting that septum formation has taken place in almost all immature, expanding chlamydospores by this time.

The maturation process of chlamydospores of *P. ramorum* reveals a strong relationship with wall thickness and diameter; wall thickness tends to increase as diameter increases (Figures 18 & 19). This phenomenon can also be seen among other *Phytophthora* species. Other chlamydospore-producing *Phytophthora* species that produce larger chlamydospores tend to have thicker walls and species that produce smaller chlamydospores tend to have thinner walls (r^2-value = 0.27) (Figure 20). This relationship is most pronounced in the species that produce chlamydospores with walls > 1.5 µm (Figure 20, largest ellipse). The trend is less pronounced in species with walls < 1.5. Some thin-walled species produce large chlamydospores such as *P. lateralis* and *P. insolita* (Figure 20 P & O). *P. cinnamomi* (Figure 20 L) is generally considered a thin-walled species but average wall thickness values recorded for the species place it in an intermediate category under the current definition; it too produces large diameter chlamydospores. Other species produce small diameter, relatively thick-walled

chlamydospores. *P. drechsleri* (Figure 20 K) has the smallest average diameter chlamydospores for the genus, but has an intermediate wall thickness (1.3 µm).

Figure 20: Chlamydospore Diameter and Wall Thickness Relationship among *Phytophthora* Species



Diameter vs. Wall thickness in *Phytophthora*: (A) *P. undulata* (B) *P. macrochlamydospora* (C) *P. colocasiae* (D) *P. ramorum* (E) *P. parasitica* (F) *P. capsici* (G) *P. boehmeriae* (H) *P. palmivora* (I) *P. meadii* (J) *P. cactorum* (K) *P. drechsleri* (L) *P. cinnamomi* (M) *P. arecae* (N) *P. citrophthora* (O) *P. insolita* (P) *P. lateralis* (Q) *P. megasperma* (R) *P. polygoni* (S) *P. tentaculata*. References for diameter and wall thickness values can be found in Appendix A.

Results presented here provide evidence that in most chlamydospores of *P. ramorum* expansions ceases after formation of a delimiting septum (Figure 16). Cytological observations on chlamydospore expansion have revealed that *Phytophthora* chlamydospores expand with a thin wall (i.e. < 1.0 µm) (Hemmes & Wong 1975; Hemmes & Lerma 1985), a result which was also seen in this research. Cultures on V8 agar (up to 31 days old) and V8JB (between 10 and 120-days- old) produced chlamydospores with walls < 1.0 µm and having diameters that ranged from 19.0-56.0 µm and 18.0-59.5 µm respectively (Figures 18 & 19). In both V8 agar and V8JB-grown cultures, more than 1/3 of the chlamydospores recorded in this thesis had diameters greater than the maximum diameters for expanding thin-walled chlamydospores (Figures 18 & 19). V8 agar-grown chlamydospores with wall thicknesses ≥ 1.0 µm ranged in wall thickness from 1.0-4.4 µm and in diameter from 56.0-80.5 µm; V8JB chlamydospores had wall thicknesses from 1.0-5.0 µm and diameters from 59.5-81.8 µm (Figures 18 & 19).

This phenomenon could be caused by several factors: (1) Chlamydospore expansion occurs more quickly in younger cultures, likely due to rapid growth of the fungal colony before nutrients become depleted. The chlamydospores from cultures on V8 agar were sampled only after 31 days, by which time the colony had covered the entire agar surface. The V8JB-grown cultures were sampled at 10, 20, 30, 60, 90, and 120 days of age. The nutrients in these cultures were likely exhausted of resources as well at time of sampling. Chlamydospore formation continues to occur in the culture as available resources are being exhausted by the colony as it grows, less cytoplasm is produced due to less uptake of nutrients, and chlamydospore expansion rates decrease with the overall effect that newer thin-walled chlamydospores expand less in older cultures than in younger ones. (2) Chlamydospore expansion may continue after the formation of the septum. It is possible that some chlamydospores of *P. ramorum* continue to expand at the same time that they are depositing a thicker inner wall. This is unlikely, however, based on what is known about thin expanding walls and the later deposition of the inner thicker wall. TEM photographs have clearly shown that the two walls are different and distinct in other *Phytophthora* species (Hemmes & Lerma 1985). (3) It is possible that the sample of the thinner-walled chlamydospores simply did not contain larger diameter chlamydospores. This is unlikely due to the large number of chlamydospores that were sampled that had larger diameters. The most likely explanation is that cultures of *P. ramorum* that have access to unlimited resources produce larger chlamydospores than cultures that are limited in resources.

There has been very little research done on the development of chlamydospores in the genus *Phytophthora*. The work that has been done has focused largely on cytological analyses of the chlamydospore cytoplasm composition, chlamydospore wall structure, and septum structure (Hemmes & Lerma 1985). These studies have provided a detailed picture of development at distinct stages of chlamydospore development. This is the first study to follow the development of the chlamydospore wall and diameter continuously through time in the genus *Phytophthora* and with *P. ramorum*. This is the first time that a comparison of chlamydospore wall thickness and diameter has been done across the entire genus of *Phytophthora*. This is also the first time that the relationship between chlamydospore wall thickness and diameter has been described within *P. ramorum* and across *Phytophthora* species. These experiments in chlamydospore development represent an important first step in understanding the role of the chlamydospore in *P. ramorum* and across all chlamydospore-producing *Phytophthora* species.

Chapter 2 Cited Literature

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Chapter 3

Biology of Chlamydospores of *Phytophthora ramorum*: Chlamydospore Germination

Aaron Smith

Introduction

The chlamydospore is an important survival propagule in *Phytophthora* which allows the fungus to survive periods not conducive to vegetative growth. When the appropriate conditions are encountered the chlamydospore is able to germinate and form a new clone of the parent. Germination allows the fungus to infect host material or form new colonies on agar through vegetative growth or through the formation of sporangia and subsequent zoospore release. The conditions that allow for chlamydospore germination to occur are varied among *Phytophthora* species, but there are some factors that are consistent to all species and others that are not well understood.

An important factor in chlamydospore germination research is the determination of chlamydospore viability. There have been two main methods used to determine chlamydospore viability: dye exclusion staining with stains such as rose bengal and direct observation of chlamydospore germination. The use of the stain rose bengal was used primarily in research dating to the 1960s. The method involves treatment of a sample chlamydospore suspension (known to contain both viable and dead chlamydospores) with aqueous rose bengal solution for 6-12 hours in the darkness at 24 C. Injured and nonviable chlamydospores selectively stain red, but viable chlamydospores remain unstained. Viability is therefore based on the integrity of the chlamydospore cell membrane to exclude the dye. The number of viable chlamydospores in the chlamydospore suspension are then used to calculate the percent of germinated chlamydospores. One limitation of the procedure was elucidated by Mircetich *et al*. (1968). They found that chlamydospore suspension comprised of chlamydospores grown in media containing citric acid or V8 juice amended with streptomycin sulfate, could not be selectively stained by rose bengal (Mircetich *et al*. 1968). Preliminary research from this thesis revealed that *P. ramorum* V8JB-grown chlamydospores were also not affected by treatment with rose bengal. In chlamydospore suspensions that were known to contain both viable and dead (primarily due to mechanical damage sustained in the process used to separate them from hyphae) chlamydospores, viable chlamydospores could not be reliably distinguished using the rose bengal method of Mircetich *et al*. (1968). The second method for determination of viability is done by observing germination of the chlamydospore. This is the most reliable and unambiguous method. However, a significant difficulty is that it is impossible to determine whether an ungerminated spore is viable at the time it is observed. It is possible that an ungerminated spore is viable, but has not yet germinated. The use of this method could therefore result in an underestimate of chlamydospore viability, but it remains the most reliable determination of chlamydospore viability.

Phytophthora chlamydospores generally require an aqueous environment to germinate, although some species are able to germinate at relatively low matric potentials (dry conditions). All species studied germinate on agar surfaces. Chlamydospores of some species germinate in water, but

rates of germination are relatively low in most species. *P. cinnamomi* chlamydospores germinated in demineralized water at a rate of 6 % (Mircetich *et al*. 1968). Observations in this thesis of germination of *P. ramorum* chlamydospores indicate that germination occurs in DIH_2O, but that germination rates are extremely low. In contrast, chlamydospores of *P. palmivora* readily germinate in water with germination rates as high as 95 % (Kadooka & Ko 1973). Some species will germinate under relatively dry conditions as well. Chlamydospores of *P. cinnamomi* had 70 % germination at 0 to -0.1 bar with germination decreasing to 40-50 % at -0.25 bar (Sterne *et al*. 1977). Chlamydospore germination occurred in *P. drechsleri* at an osmotic potential as low as -97.3 bars with 26.6 % germination after 48 hours incubation, but no germination occurred at -110 bars (Cother & Griffin 1974).

Temperature has varying effects on chlamydospore germination in different species. Chlamydospores of *P. cinnamomi* germinate over a wide range of temperatures ranging from 9 to 36 C with an optimum germination temperature of between 20-30 C (Mircetich *et al.* 1968). In some species, chlamydospore germination is controlled by pre-germination exposure to cold temperatures. Chlamydospores of *P. cactorum* only germinated after freezing at -23 C for 24 hours with subsequent germination rates of 60-80 % upon thawing (Darmono & Parke 1990). The range of temperatures under which chlamydospore germination occurs in *P. ramorum* is not known.

The effect of pH on chlamydospore germination also varies by species. Mircetich *et al*. (1968) found that chlamydospores of *P. cinnamomi* germinated over a wide pH range, with optimal germination occurring between pH 5 and 7, although over 55 % of chlamydospores germinated between pH 3 and 9. No germination occurred at pH 2.5. In chlamydospores of *P. palmivora* the pH range where germination occurred was much narrower. Chee (1973) reported that no chlamydospores germinated at pH 4 or 8 but optimal germination occurred at 5.8. The effect of pH on the germination of chlamydospores of *P. ramorum* is not known.

Dormancy of chlamydospores in *Phytophthora* is exogenous, meaning that there is a requirement of a stimulus from the environment, such as presence of water or nutrients, to break dormancy (Sussman 1966). Of the species that have been studied, only *P. palmivora* has high rates of germination in water. Kadooka & Ko (1973) reported 82 % germination of thin-walled *P. palmivora* chlamydospores in demineralized water. Most species require an exogenous source of nutrients to induce germination. Chlamydospores of *P. cinnamomi* germinated at a rate of only 6 % in demineralized water, whereas the addition of any of the 11 amino acids tested stimulated germination. The greatest effect was seen with the addition of asparagine (0.125 M), which resulted in 83 % germination (Mircetich *et al*. 1968). Alizadeh & Tsao (1985) found that germination of *P. cinnamomi* chlamydospores varied depending on growth media. After 10 hours of incubation at 25 C germination rates of 65 % on carrot broth, 73 % on V8 broth, 57 % on glucose and asparagine, and 54 % on asparagine alone were observed. Khan & Reeleder (1996) reported variation in chlamydospore

germination in *P. cactorum* from 5 to 30 % depending on medium type and temperature. Chlamydospores of *P. parasitica* had 38-49 % germination in 2% natural soil extract, but no germination in distilled water (Tsao & Bricker 1968).

A relationship between the thickness of the chlamydospore wall and rates of chlamydospore germination has been reported for some species of *Phytophthora*. The best evidence for the effect of wall thickness comes from Kadooka & Ko (1973) who found higher rates of germination (95 %) for thin-walled (< 1.0 µm) chlamydospores of *P. palmivora* on V8 juice agar, compared to only 5 % germination for thick-walled (> 1.5 µm) chlamydospores. Furthermore, on water agar, germination of both thin-walled and thick-walled chlamydospores was between 90 and 95 %, whereas in demineralized water, germination was 17 % for thick-walled and 82 % for thin-walled chlamydospores (Kadooka & Ko 1973).

The age of the chlamydospore has varied effects on germination of chlamydospores of *Phytophthora*. In chlamydospores of *P. cinnamomi* (Mircetich *et al*. 1968) and *P. parasitica* (Holdaway & Tsao 1972), the age of the chlamydospore appeared to have no effect on chlamydospore germination. In *P. palmivora,* one-week-old chlamydospores germinated at a rate of 95 %, which decreased to 81 % at 40-days-old, 35 % at 80-days-old, and showed no germination in 120-day-old chlamydospores (Chee 1973). The effect of chlamydospore age on germination is not well understood in *P. ramorum*.

Duration of incubation appears to have an effect on the germination of chlamydospores of some species. Chlamydospores of *P. drechsleri* had no germination after 24 hours but had 26 % germination after 48 hours of incubation on V8 juice agar sealed in plastic bags at 25 +/- 1 C (Cother & Griffin 1974). Ioannou & Grogan (1985) found that chlamydospores of *P. parasitica* germinated on CMA after 12, 18, and 24 hours of incubation in uncovered Petri dishes at 22 C in the dark. Chlamydospores of *P. palmivora* germinated within ten hours of incubation at 26 C in water at 50-60 % germination rates (Hemmes & Lerma 1985).

The objectives of the experiments presented here were to understand some of the factors that affect the germination of *P. ramorum* chlamydospores. This was accomplished by: (1) characterizing chlamydospore germination in *P. ramorum*; (2) measuring the effects of different nutrient media on chlamydospore germination; (3) comparing the diameters and wall thicknesses of germinated chlamydospores and non-germinated viable chlamydospores; (4) comparing germination rates of chlamydospores of different ages; and (5) measuring the number of chlamydospores that germinate over time.

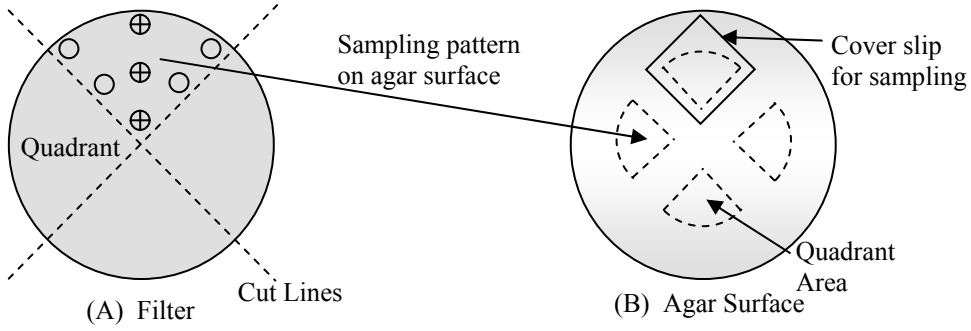
Methods

Separation of Chlamydospores

P. ramorum isolate 2027.1 was used except as otherwise noted. Cultures were grown and stored as described in Chapter 2 (Chlamydospore Formation). Chlamydospores were extracted from cultures grown in V8JB by maceration and filtration. All components were sterilized by autoclaving. Fungal mats from four V8JB cultures were placed on a Miracloth filter inside a 186 ml Buchner funnel (CoorsTek®). Hyphal mats were rinsed with sterile DIH_2O until they were white. The rinsed mats were transferred to a #200 sieve on a catch pan (Braun-Knecht-Heimann Co., San Franscisco, 100 µm filter) and rinsed 3x for 20 s with sterilized DIH_2O. Between each rinse the mats were spread across the surface of the sieve with a rubber policeman to aid separation of chlamydospores from the hyphae. The chlamydospore suspension was then transferred from the catch pan to a beaker and 400 ml autoclaved DIH_2O was added.

Chlamydospores were transferred to agar plates after collection on 5.0 µm micropore polycarbonate filters (Whatman, PC MB 47 mm 5.0 µm). The chlamydospore suspension was stirred and 100 ml was aspirated through the filter. The filter with chlamydospores was then cut into four quadrants [1/2 π rad] with the aid of a sterilized razor blade (Figure 21 A). Each filter quadrant was placed with the chlamydospore-bearing side in contact with the agar surface in a Petri dish then immediately peeled away to transfer the chlamydospores from the filter to the agar surface. This process was repeated until each Petri dish contained four quadrant areas (Figure 21 B). The Petri dishes were sealed with Parafilm® and incubated for 24 hours at 20 C in the dark.

Figure 21: Filter, Quadrant Placement, and Sampling Pattern



⊕ Germinated, intact, and broken chlamydospores sampled.
○ Germinated chlamydospores sampled.

Chlamydospore Germination

Intact chlamydospores were defined as chlamydospores that did not have any sign of chlamydospore wall rupture (Figure 24 A-C) or unnatural disturbance pattern in the cytoplasm (Figure 24 B & C). Normal cytoplasm contains numerous small, evenly dispersed granular inclusions (Figure 24 B) or a large central vacuole is present with smaller scattered granular inclusions (Figure 24 C). Broken chlamydospores were defined as chlamydospores that had signs of chlamydospore wall rupture or an abnormal appearing cytoplasm (Figure 24 D-F). The cytoplasm was considered abnormal if there was apparent aggregation of organelles or cytoplasmic inclusions (Figure 24 D). Chlamydospores were considered to have germinated if the wall was intact and had two or more germ tubes equal to or larger than the diameter of the chlamydospore (Figure 25 B & C, Figure 26 A-C) or at least one germ tube equal to or larger than the diameter of the chlamydospore (Figure 25 A).

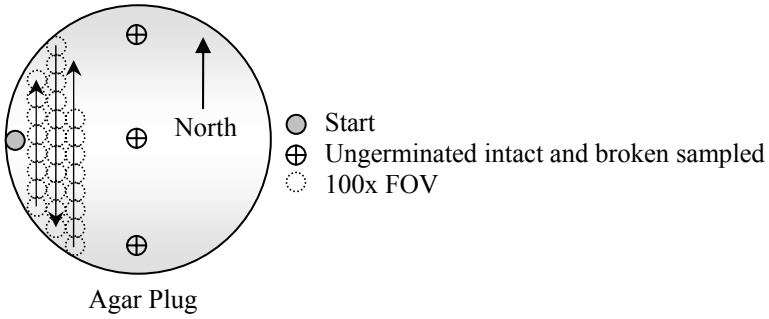
Calculation of Percent Germination and Mean Chlamydospore Wall Thickness, Diameter, and Germination: Percent germination was calculated by the formula: % G = G / I + G (Where G = Number of germinated chlamydospores observed, and I = Average number of intact but ungerminated chlamydospores multiplied by the number of Fields of View (FOV) sampled. Data were entered into Microsoft® Excel 2002. Mean chlamydospore wall thickness, diameter, and germination were calculated using S-plus® 7.0 statistical software by calculating the mean of the measured values; the associated ranges of values were constructed by minimum and maximum values. Data were subjected to chi-square goodness of fit tests with a Yates' correction and Analysis of Variance (ANOVA) as appropriate.

Chlamydospore Germination on Different Media (Germination Experiment A): Chlamydospore germination was compared on three different media: V8 agar, cornmeal agar with antibiotics (CAR), and water agar (WA) without antibiotics (Appendix B). Each replication consisted of one Petri dish of each medium (three Petri dishes), with four quadrants of the same filter per plate, with two replications. The experiment was performed twice (Experiments one and two). On the bottom of each Petri dish seven sample points were marked under each of the quadrant areas (Figure 21 A). After 24 hours incubation the lids were removed from the Petri dishes and a drop of autoclaved DIH_2O and a cover slip were placed over one quadrant area (Figure 21 B); drops of autoclaved DIH_2O and cover slips were added to each quadrant separately at time of sampling. Chlamydospores were counted on the surface of the agar at 100x magnification with a Zeiss compound microscope. At three sample points (Figure 21 A) for each quadrant area, all intact but ungerminated, broken, and germinated chlamydospores were tallied in a 100x (4.2 mm^2) FOV under bright field illumination. At the remaining four points in each quadrant area, only germinated chlamydospores were tallied (Figure 21 A). Only

chlamydospores that had their entire diameter inside the outer circumference of the FOV were included in the sample.

Chlamydospore Germination on V8 and WA (Germination Experiment B): Chlamydospore germination on V8 agar and WA were compared. Methods were similar to Germination Experiment A, except that two quadrants of one filter were placed on V8 agar and two were placed on WA agar in each of four replicate plates, and agar plugs, with chlamydospores, were preserved in formaldehyde; the experiment was performed once. After 24 hours of incubation circular 14 mm diameter plugs were removed with a #8 cork borer and placed in glass vials with formaldehyde between one and 12 days until sampled. Plugs were placed with the chlamydospore-bearing surface up on a microscope slide and covered with a cover slip. Chlamydospores were counted in sequential 100x FOV (Figure 22) across the entire plug starting with the western edge of the plug so that only the agar surface was seen in the FOV. Germinated chlamydospores in each of the FOV were tallied. An average of 32 FOV were thus tallied for each quadrant area.

Figure 22: Agar Plug Sampling Pattern



Wall Thickness and Diameter of Germinated and Ungerminated Intact Chlamydospores (Germination Experiment C): Wall thickness and diameter of germinated and intact but ungerminated chlamydospores were compared with the same chlamydospores measured in Germination Experiment B. Methods and sampling were similar to those used in Germination Experiment B, except that wall thickness and diameter of each germinated chlamydospore and the nearest ungerminated chlamydospore were measured at 1000x under bright field illumination. The next germinated chlamydospore to the north and the nearest intact, ungerminated chlamydospore to it were located and then measured. This procedure was repeated until the entire plug or 25 germinated and 25 intact but ungerminated chlamydospores had been located and measured (Figure 22).

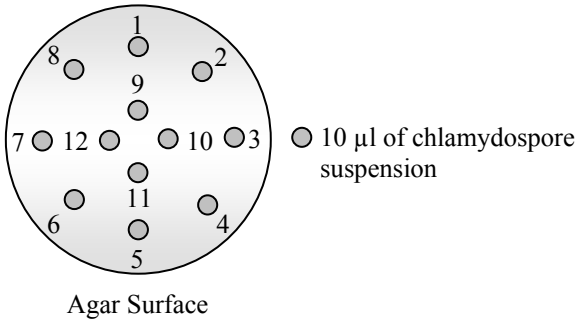
Wall Thickness and Diameter of Processed and Unprocessed V8JB culture Chlamydospores (Germination Experiment D): Wall thickness and diameter of "processed" (chlamydospores separated from hyphae following the procedure outlined under "Separation of Chlamydospores" above

and placed on the agar surface following the procedure used in Germination Experiment B) germinated and intact, and ungerminated intact chlamydospores taken directly from "unprocessed" V8JB cultures were compared. Preparation for germination was similar to Germination Experiment B and sampling methods were similar to Germination Experiment C except four quadrants of one filter were placed on one plate of V8 agar per replication. The experiment was performed once. After the 24 hours of incubation, plugs were removed and stored in formaldehyde in glass vials for five to nine days until sampled. Chlamydospores were sampled as above (Figure 22) until 50 germinated chlamydospores were sampled across multiple plugs. For comparison, samples were taken directly from the same V8JB cultures that the germinated chlamydospores had originated from and placed in glass vials with formaldehyde for one to six days until sampled. In each replication, 50 V8JB chlamydospores were sampled as in the "Chlamydospore Maturation in V8JB" experiment (See Chapter 2, Figures 14 & 16).

Chlamydospore Germination and Age of Culture (Germination Experiment E): Chlamydospore germination was compared for chlamydospores from V8JB cultures aged 10, 90, and 170 days. Chlamydospores were sampled according to the procedures in the Germination Experiment B (Figure 22) except that all four quadrants of the filter were placed on each V8 agar plate in each age class and replication. Plugs were stored in formaldehyde from between three to six days until sampled. The experiment was performed once.

Chlamydospore Germination Over Time (Germination Experiment F): Chlamydospore germination was observed in two trials for periods of 26 and 19 days duration, respectively, on V8 agar with antibiotics (Appendix B, V8ARP). Each trial consisted of 60 and 30 Petri dishes respectively, with three replications each, and the experiment was performed twice (Experiments one and two respectively). Separation of chlamydospores from mycelium was similar to methods described above under "Separation of Chlamydospores" except that for each replication the chlamydospore suspension was adjusted to a concentration of one chlamydospore per 10 µl with autoclaved DIH_2O. One 10 µl aliquot was pipetted onto the agar surface at each of 12 sample points (Figure 23), covered with a lid, and sealed with Parafilm®. The Petri dishes were evaluated for germination every one to three days by initial inspection with the unaided eye. Germination was then confirmed by observation under the compound microscope at 100x magnification. Newly germinated chlamydospore colonies were cut from the agar surface. A positive control was prepared following the chlamydospore germination protocol with 100 ml of each replication's adjusted chlamydospore suspension on V8 agar.

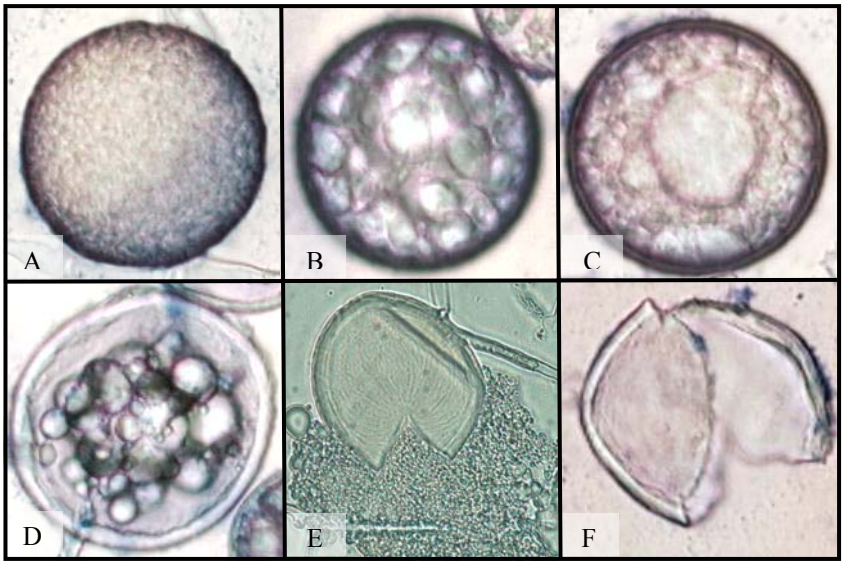
Figure 23: Sampling Pattern for Chlamydospore Germination Over Time.



Results

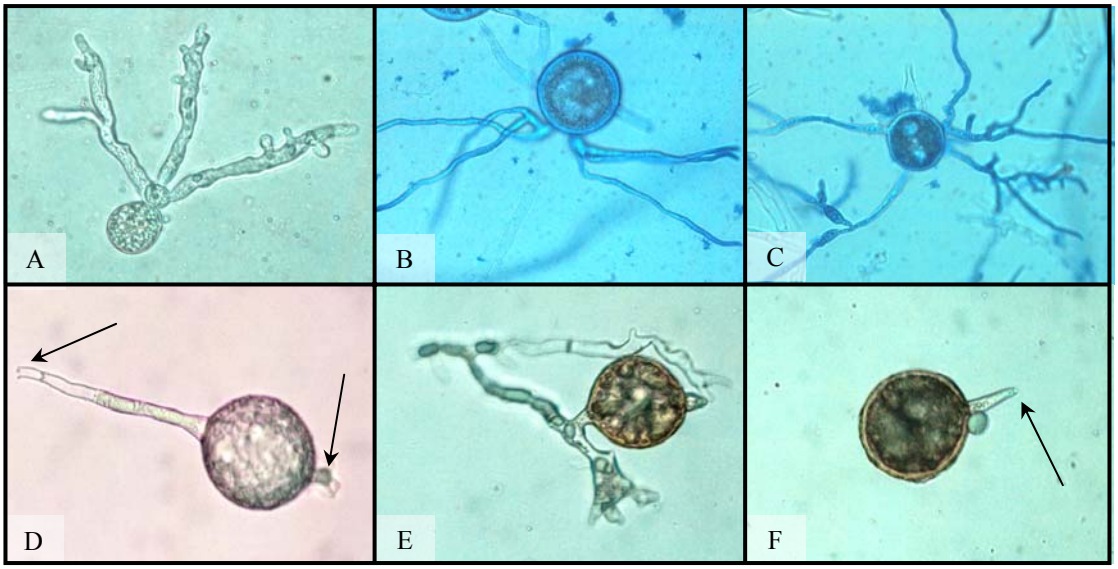
Chlamydospore Germination

Figure 24: Comparison of Intact and Broken *P. ramorum* Chlamydospore Morphology



Intact and Broken Chlamydospore Morphology: (A) Intact without visible vacuoles. (B) Intact with visible evenly dispersed granular inclusions. (C) Intact with large central vacuole and small scattered granular inclusions (periphery). (D) Broken with aggregated organelles and cytoplasmic inclusions. (E) Broken with ruptured wall and cytoplasm (bottom). (F) Broken with ruptured wall. *P. ramorum* isolate P-110 chlamydospores on V8 agar at 400x under bright field illumination. A-D and F treated with Aniline blue stain, E shown immersed in DIH_2O.

Figure 25: Comparison of Early Chlamydospore Germination and Non-germination



Early Chlamydospore Germination and Non-germination: (A) Germinated with single germ tube longer than diameter of chlamydospore. (B) Germinated with two germ tubes longer than the diameter of the chlamydospore. (C) Germinated with four germ tubes longer than the diameter of the chlamydospore. (D) Non-germinated with two broken connecting hyphal fragments (arrows). (E) Non-germinated with large subtending hyphal fragment with partial cytoplasm. (F) Non-germinated with single broken subtending hyphal fragment (arrow). *P. ramorum* isolate 2027.1 (A, D-F) and isolate P-110 (B & C) chlamydospores on V8 agar at 100x under bright field illumination. A and D-F are shown immersed in DIH_2O, B and C are treated with Aniline blue stain. Photographs are enlarged to clarify characteristics.

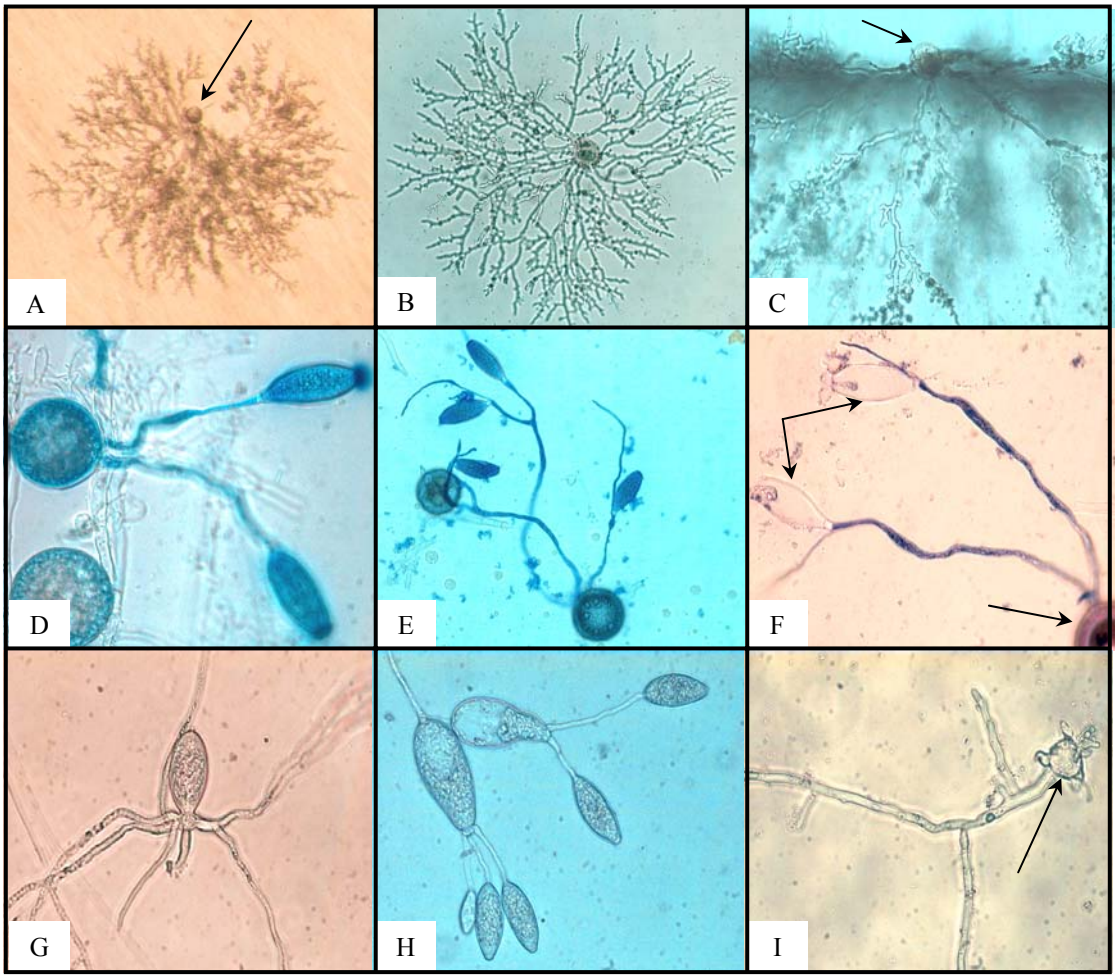
General Chlamydospore Germination and Subsequent Asexual Reproduction and Growth:

Results from preliminary experiments indicated that some chlamydospores of *P. ramorum* isolate 2027.1 germinated within 8-10 hours of plating on V8 agar when incubated at 20 C in the dark. By 22 hours single germ tube growth averaged 309 µm (n = 8). Within 24 hours of plating on agar, the hyphae of many germinated chlamydospores of *P. ramorum* isolates P-110 and 2027.1 were visible to the unaided eye; under 100x magnification finer details of the chlamydospore germination could be discerned (Figure 25 A-C, Figure 26 A-C). Chlamydospores germinated with one (Figure 25 A) or multiple germ tubes (Figure 25 B & C, Figure 26 A-C). Preliminary results indicated that germinated chlamydospores could be distinguished from ungerminated chlamydospores that were still attached to hyphal fragments by determining whether apparent germ tubes contained cytoplasm and had rounded (Figure 25 A) tips (i.e. not broken or injured tips, Figure 25 D, E, & F). Chlamydospores were tallied as "germinated" if multiple germ tubes or a single branched germ tube were present. Chlamydospores with a single germ tube that had not yet branched at the time of sampling were only considered germinated if the germ tube was at least as long as the diameter of the source chlamydospore.

Chlamydospores with a simple, short, hyphal papilla were tallied as "ungerminated" (Chapter 2, Figure 7 I, M-N, P-S, Z, & BB).

P. ramorum chlamydospore germ tubes grew and branched rapidly (Figure 25 A-C, Figure 26 A-C) forming diagnostic warty hyphae especially on V8 agars (V8 & V8ARP). This was less pronounced on cornmeal (CMA & CAR) or water agar (WA) (Appendix B). New germ tubes formed on the side of the chlamydospore that was in contact with the agar surface, grew first into the agar (Figure 26 C), and then grew radially outward (Figure 26 B). Chlamydospores were also seen to germinate to produce single or multiple sporangia (Figure 26 D-F), especially on WA (Figure 26 E & F) and less often on cornmeal agar. Germination directly to form sporangia was least frequent on V8 agars. Sporangia produced from germinated chlamydospores were often empty and were presumed to have released their zoospores prior to observation (Figure 26 F). Germ tubes produced on WA and cornmeal agars tended to be thinner (Figure 26 D-F) than germ tubes produced on V8 agars (Figure 25 A). Germ tubes were most branched on V8 agars (Figure 26 A-C), less branched on cornmeal agars, and least branched on WA (Figure 26 E & F). Sporangia were seen to germinate directly to germ tubes (Figure 26 G) and to other sporangia (Figure 26 H). Hyphal swellings often exhibited vegetative growth that very closely resembled growth of germ tubes from germinated chlamydospores (Figure 26 I). Some hyphal fragments that contained intact cytoplasm were observed to grow vegetatively on the agar surface.

Figure 26: *P. ramorum* Colony-forming Chlamydospore Germination and Subsequent Asexual Reproductive Growth



Chlamydospore Germination and Subsequent Growth: (A) Germinated chlamydospore (arrow) and colony (Stereo microscope view). (B) Germinated chlamydospore and colony (at 100x). (C) Side view of germinated chlamydospore (arrow) on agar surface with germ tubes growing into and along agar surface (at 200x). (D) Germinated chlamydospore (left) with two germ tubes ending in two sporangia (right) (E) Germinated chlamydospore with three germ tubes and multiple sporangia. (F) Germinated chlamydospore (right arrow) with two sporangia with their zoospores released (left arrows). (G) Germinated sporangia to multiple germ tubes. (H) Germinated sporangia to multiple germ tubes, each with a sporangium. (I) Vegetative growth from hyphal swelling (arrow). *P. ramorum* isolate 2027.1 (A-C) and isolate P-110 (D-I) chlamydospores (A-F) and sporangia (G & H) 24 hours after plating on V8 agar under bright field illumination. A and C are shown without treatment on agar surface; B, G, and I are shown immersed in DIH_2O; D-F and H are treated with Aniline blue stain.

Chlamydospore Germination on Different Media (Germination Experiment A): P. ramorum chlamydospore germination was significantly higher on V8 agar (362 chlamydospores) than CAR (75 chlamydospores) (chi-square = 187.2, df = 1, $p < 0.001$) or WA (56 chlamydospores) (chi-square = 222.5, df = 1, $p < 0.001$). Chlamydospore germination was not significantly different on CAR than on WA (chi-square = 2.5, df = 1, $p = 0.2 < p < 0.1$). This corresponded to a percent germination of 10.7 % on V8 agar, 2.4 % on CAR, and 1.7 % on WA (Table 5, Experiment 1, All Replications). In experiment two, chlamydospore germination was also significantly higher on V8 agar (187 chlamydospores) than CAR (17 chlamydospores) (chi-square = 140, df = 1, $p < 0.001$) or WA (26 chlamydospores) (chi-square = 120.2, df = 1, $p < 0.001$). Chlamydospore germination was not significantly different on CAR than on WA (chi-square = 1.5, df = 1, $p = 0.3 < p < 0.2$). This result corresponded to a percent germination of 4.3 % on V8 agar, 0.5 % on CAR, and 0.6 % on WA (Table 5, Experiment 2, All Replications).

Table 5: Number and Percent of Germinated Chlamydospores on Different Media

Experiment	Replication	Treatment	Number of Germinated Chlamydospores (Range / 100x FOV)	Percent Germination
Experiment 1	All Replications	V8	362 (0-18)	10.7 %
		CAR	75 (0-5)	2.4 %
		WA	56 (0-4)	1.7 %
	Replication 1	V8	126 (0-10)	8.1 %
		CAR	26 (0-3)	1.5 %
		WA	26 (0-3)	1.6 %
	Replication 2	V8	236 (2-18)	12.9 %
		CAR	49 (0-5)	3.6 %
		WA	30 (0-4)	1.9 %
Experiment 2	All Replications	V8	187 (0-8)	4.3 %
		CAR	17 (0-3)	0.5 %
		WA	26 (0-3)	0.6 %
	Replication 1	V8	67 (0-6)	3.1 %
		CAR	8 (0-3)	0.6 %
		WA	12 (0-2)	0.6 %
	Replication 2	V8	120 (0-8)	5.6 %
		CAR	9 (0-2)	0.5 %
		WA	14 (0-3)	0.6 %

Chlamydospore Germination on V8 and WA (Germination Experiment B): P. ramorum chlamydospore germination was significantly higher on V8 agar (467 chlamydospores) than on WA (74 chlamydospores) (chi-square = 284, df = 1, $p < 0.001$). This corresponded to a percent germination of 5.6 % on V8 agar and 1.5 % on WA (Table 6, All Replications).

Table 6: Numbers and Percent of Germinated Chlamydospores on V8 and WA

Replication	Treatment	Number of Germinated Chlamydospores (Range / 100x FOV)	Percent Germination
All Replications	V8	467 (0-14)	5.6 %
	WA	74 (0-4)	1.5 %
Replication 1	V8	96 (0-9)	4.4 %
	WA	46 (0-4)	4.0 %
Replication 2	V8	68 (0-6)	3.8 %
	WA	2 (0-1)	0.3 %
Replication 3	V8	93 (0-9)	9.1 %
	WA	6 (0-1)	0.5 %
Replication 4	V8	210 (0-14)	7.0 %
	WA	20 (0-2)	1.4 %

Wall Thickness and Diameter of Germinated and Ungerminated Intact Chlamydospores (Germination Experiment C): There was no significant difference between germinated mean chlamydospore wall thickness (1.6 µm) and ungerminated mean intact wall thickness (1.5 µm) ($p = 0.07$) (Table 7, All Replications).

	df	SS	MS	F	P
V8 or WA	1	0.0352	0.0352	0.1575	0.6917
Chlm. Wall Thickness	1	0.7451	0.7451	3.3361	0.0685
Residuals	417	93.1328	0.2233		

Germinated mean diameter was significantly smaller (43.6 µm) than ungerminated intact diameter (50.5 µm) ($p = < 0.0001$) (Table 7, All Replications).

	df	SS	MS	F	P
V8 or WA	1	1.34	1.344	0.0166	0.8977
Chlm. Diameter	1	5059.61	5059.607	62.3409	0.0000
Residuals	417	33843.86	81.160		

V8 and WA had no significant effect on germinated or ungerminated intact wall thickness ($p = 0.7$) or diameter ($p = 0.9$).

Table 7: Mean Germinated and Ungerminated Intact Chlamydospore Wall Thickness and Diameters

Treatment	All Replications Mean for V8 and WA Treatments Combined (Range) (µm)
Germinated Wall Thickness	1.6 (0.8-3.0)
Ungerminated Intact Wall Thickness	1.5 (0.8-4.1)
Germinated Diameter	43.6 (20.0-85.0)
Ungerminated Intact Diameter	50.5 (23.5-70.0)

	Replication 1 Mean V8 (Range) Mean WA (Range) (µm)	Replication 2 Mean V8 (Range) Mean WA (Range) (µm)	Replication 3 Mean V8 (Range) Mean WA (Range) (µm)	Replication 4 Mean V8 (Range) Mean WA (Range) (µm)
Germinated Wall Thickness	1.7 (0.9-2.5) 1.4 (0.8-2.3)	1.6 (0.9-3.0) 0.9 (1.8-1.8)	1.7 (1.0-3.0) 1.6 (1.8-2.4)	1.6 (0.9-3.0) 1.8 (1.3-2.4)
Ungerminated Intact Wall Thickness	1.5 (0.8-2.5) 1.5 (0.9-2.5)	1.5 (1.0-2.5) 0.8 (1.5-1.5)	1.5 (0.9-2.9) 2.0 (1.5-4.1)	1.5 (0.8-2.6) 2.0 (1.5-2.5)
Germinated Diameter	46.3 (33.0-69.0) 42.3 (20.0-85.0)	42.8 (23.0-61.0) No Data	41.0 (22.5-60.0) 51.5 (42.0-69.0)	42.4 (28.8-57.0) 51.2 (40.5-64.5)
Ungerminated Intact Diameter	48.3 (35.0-60.0) 48.2 (29.0-64.3)	51.0 (36.0-68.5) No Data	51.0 (32.5-63.0) 44.4 (29.0-68.8)	52.7 (23.5-70.0) 51.4 (40.0-68.0)

Wall Thickness and Diameter of Processed and Unprocessed V8JB culture Chlamydospores (Germination Experiment D): Germinated mean chlamydospore wall thickness (processed) was significantly thinner (1.5 µm) than parent V8JB culture mean chlamydospore wall thickness (1.8 µm) (unprocessed) (p = < 0.0001) (Table 8, All Replications).

	df	SS	MS	F	P
Chlm. Wall Thickness	1	6.3378	6.3378	25.7167	0.0000
Residuals	398	98.0861	0.2464		

Germinated mean chlamydospore diameter (processed) was significantly smaller (41.7 µm) than parent V8JB culture mean chlamydospore diameter (55.4 µm) (unprocessed) (p = < 0.0001) (Table 8, All Replications).

	df	SS	MS	F	P
Chlm. Diameter	1	18820.41	18820.41	199.3375	0.0000
Residuals	398	37577.08	94.41		

Table 8: Mean Germinated and V8JB Chlamydospore Wall Thickness and Diameters

Treatment	All Replications Mean (Range) (µm)
Germinated Wall Thickness (Processed)	1.5 (0.6-4.5)
V8JB Wall Thickness (Unprocessed)	1.8 (0.5-3.5)
Germinated Diameter (Processed)	41.7 (21.5-69.0)
V8JB Diameter (Unprocessed)	55.4 (19.5-83.8)

Treatment	Replication 1 Mean (Range) (µm)	Replication 2 Mean (Range) (µm)	Replication 3 Mean V8 (Range) (µm)	Replication 4 Mean V8 (Range) (µm)
Germinated Wall Thickness (Processed)	1.5 (0.9-2.5)	1.5 (0.8-4.5)	1.5 (0.6-2.5)	1.6 (0.6-3.3)
V8JB Wall Thickness (Unprocessed)	1.6 (0.6-3.5)	1.9 (0.5-3.0)	1.7 (0.8-2.5)	2.0 (1.0-2.8)
Germinated Diameter (Processed)	42.3 (21.5-67.0)	42.1 (24.8-62.0)	40.9 (22.0-69.0)	41.3 (24.0-64.5)
V8JB Diameter (Unprocessed)	60.1 (19.5-83.8)	55.0 (36.0-74.0)	55.0 (33.0-70.5)	51.4 (30.0-74.0)

Chlamydospore Germination and Age of Culture (Germination Experiment E): Chlamydospore germination was significantly higher in 170-day-old chlamydospores (1268 chlamydospores) than in 90-day-old (364 chlamydospores) (chi-square = 499.6, df = 1, $p < 0.001$) or 10-day-old chlamydospores (226 chlamydospores) (chi-square = 725.4, df = 1, $p < 0.001$). Chlamydospore germination was significantly higher in 90-day-old chlamydospores than 10-day-old chlamydospores (chi-square = 31.8, df = 1, $p < 0.001$). This corresponded to a percent germination of 9.6% in 170-day-old chlamydospores, 3.4 % in 90-day-old, and 2.2 % in 10-day-old (Table 9, All Replications).

Table 9: Numbers and Percent Germination of Chlamydospores from V8JB cultures of Different Ages

Replication	Chlamydospore Age (Days)	Number Germinated Chlamydospores / Replication (Range / 100x FOV)	Percent Germination
All Replications	10	226 (0-7)	2.2 %
	90	364 (0-7)	3.4 %
	170	1268 (0-11)	9.6 %
Replication 1	10	78 (0-7)	3.1 %
	90	13 (0-2)	0.6 %
	170	509 (1-11)	15.1 %
Replication 2	10	3 (0-1)	0.2 %
	90	0 (0)	0 %
	170	400 (0-10)	10.5 %
Replication 3	10	116 (0-7)	3.8 %
	90	231 (0-7)	7.9 %
	170	25 (0-6)	1.0 %
Replication 4	10	29 (0-3)	1.1 %
	90	120 (0-6)	3.8 %
	170	334 (0-9)	9.6 %

Chlamydospore Germination Over Time (Germination Experiment F): Chlamydospores continued to germinate in low numbers for at least five days on V8ARP agar. In experiment one, two chlamydospores germinated after 24 h and one more chlamydospore germinated after five days (0.1 %), no subsequent germination was observed for up to 26 days (Table 10). In experiment two, five chlamydospores germinated after 24 h and four more after three days (0.8 %). No subsequent germination was observed for up to 19 days (Table 10).

Table 10: V8JB-grown Chlamydospore Germination Over Time on V8ARP Agar

Experiment	Days since plating on V8ARP	Number of chlamydospores germinated	Estimated Number of Chlamydospores in Experiment (Percent Germination)
Experiment 1	1	2	2160 (0.1 %)
	3	0	
	5	1	
	7	0	
	10	0	
	12	0	
	14	0	
	26	0	
Experiment 2	1	5	1128 (0.8 %)
	3	4	
	5	0	
	7	0	
	19	0	

Discussion

Chlamydospores of *P. ramorum* germinated at a low and variable rate on water agar, cornmeal agar, and two formulations containing V8. The highest rates of germination were seen on nutrient-rich V8 agar, suggesting that chlamydospore germination is, at least in part, stimulated by the presence of exogenous nutrients. Smaller diameter and thinner-walled chlamydospores tended to germinate in greater proportions than larger and thicker-walled chlamydospores after 24 hours of incubation. Chlamydospores raised in V8JB cultures for 170 days germinated in greater proportions than 10- or 90-day-old chlamydospores. Chlamydospore germination continued to occur up to five days after plating on V8ARP media.

Percent germination of *P. ramorum* chlamydospores was low and variable. In chlamydospores aged ten days in V8JB, the highest germination achieved on V8 agar after 24 hours of incubation ranged from a high of 12.9 % (Table 5) to a low of 0.2 % (Table 9). On CAR the percent germination ranged from a high of 3.6 % to a low of 0.5 % (Table 5). On WA, percent germination ranged from 4.0 % to 0.3 % (Table 6). Among chlamydospores germinated on V8ARP, percent germination was also low with an estimated 0.1 to 0.8 % germination (Table 10). There is no previously published research on chlamydospore germination by other workers following the protocols used here, which makes direct comparisons of other *P. ramorum* germination rates difficult to make, but other workers have reported germination rates to be much higher in *P. ramorum* than is reported here. Fungal recovery (likely driven, in part, by chlamydospore germination) from infected *Rhododendron* sp. leaves was recorded at 1 % and 80 % for leaves at the soil surface after one and two weeks respectively, 80 % and 65 % for

leaves at soil and litter / soil interfaces respectively after eight weeks, and 60 % recovery from leaves in soil after six months (Fichtner *et al*. 2005, *unpublished*). Davidson *et al*. (2002) reported an average of 60 % chlamydospore germination at zero days to an average of 20 % and 50 % germination by 30 days on a moist filter and stored in water respectively. In the experiment most closely resembling methods used here, Davidson *et al*. (2002) reported average chlamydospore germination of 60 % at zero days, which is 4.5 times greater than the highest germination rate (12.9 %) (Table 5) detected in the experiments presented here. In other *Phytophthora* species, "good" chlamydospore germination is considered to be ≥ 50 % (Mircetich *et al.* 1968), which many species routinely demonstrate. It is unclear why the percent germination of *P. ramorum* presented here and those reported by other workers are so drastically different, but factors such as differences in experimental design, varying definitions of germination, and differences in the conditions under which germination occurred are likely influential.

In all experiments mean chlamydospore germination was significantly higher on nutrient-rich V8 agar than on CAR or WA (Germination Experiments A & B). Chi-square analysis has several assumptions: (1) Data are randomly sampled. (2) Measurements are independent. (3) No recorded values should be less than one. (4) No more than 20 % of the recorded values should be less than five. Due to the large number of recorded zeros in the data and having more than 20 % of the values less than five, it was necessary to sum all independent measures of the number of chlamydospores germinated for each replication and use these figures in the analysis.

Greater chlamydospore germination on nutrient-rich V8 agar is good evidence that chlamydospore germination is exogenous in *P. ramorum*, and that is it is controlled, at least in part, by environmental conditions under which germination occurs. An exogenous nutrient requirement for chlamydospore germination is common in *Phytophthora*; presence of nutrients in media or exudates from plant roots is known to enhance chlamydospore germination in *P. cinnamomi* (Mircetich *et al*. 1968), *P. cactorum* (Khan & Reedleder 1996), *P. parasitica* (Tsao & Bricker 1968), and *P. ramorum* (Smith 2007, *unpublished*). *P. palmivora* is unique in the genus in that its chlamydospores do not require the presence of exogenous nutrients for germination (Chee 1973).

Smaller diameter and thinner-walled *P. ramorum* chlamydospores are more likely to germinate than large diameter thicker-walled chlamydospores. In a comparison of diameter and wall thickness of ungerminated but intact chlamydospores and germinated chlamydospores that had been incubated for 24 hours on V8 and WA agar surfaces, germinated mean diameter was significantly smaller (43.6 µm) than ungerminated intact chlamydospores (50.5 µm) on the same agar surface, although germinated wall thickness (1.6 µm) was not significantly different than ungerminated intact wall thickness (1.5 µm) in this test (Germination Experiment C, Table 7). In a related experiment the average chlamydospore diameter and wall thickness of ten-day-old V8JB-grown chlamydospores and germinated chlamydospores taken from the same sample were compared. Germinated chlamydospore diameter was

significantly smaller at 41.7 µm compared to parent cultures with an average diameter of 55.4 µm and mean germinated wall thickness was significantly thinner at 1.5 µm compared with 1.8 µm for parent cultures (Germination Experiment D, Table 8). This average 10-day-old V8JB-grown chlamydospore diameter and wall thickness was corroborated with results from a larger sample that found ten-day-old diameters to be 53.7 µm and wall thickness 1.8 µm (Chapter 2, Tables 3 & 4). The average germinated ten-day-old V8JB-grown *P. ramorum* chlamydospore is 42.7 +/- 1.0 µm in diameter and has a wall thickness of 1.6 µm (Tables 7 & 8). This is at least 10 µm smaller in diameter with a wall thickness of at least 0.2 µm thinner than the average V8JB-grown chlamydospore from which the sample was drawn. This is the first time that a description of the diameter and wall thickness of the average germinated chlamydospore has been documented in *P. ramorum*.

The average ten-day-old V8JB-grown germinated chlamydospore in *P. ramorum* is comparatively large in diameter (i.e. > 35 µm) and thick-walled (i.e. > 1.5 µm) and its rate of germination is low. Kadooka & Ko (1973) reported that thin-walled chlamydospores (< 1.0 µm) of *P. palmivora* germinated at a rate of 95 % on V8 juice agar, whereas thick-walled chlamydospores (> 1.5 µm) germinated at a rate of only 5 %. Furthermore, chlamydospore germination in demineralized water was 17 % for thick-walled chlamydospores vs. 82 % for thin-walled (Kadooka & Ko 1973). This pronounced difference in rates of germination was attributed to the effect of the thicker chlamydospore wall. Thick-walled chlamydospores germinate at a lower proportion than do thinner-walled chlamydospores. This result is consistent with the findings reported here. It is likely that the low germination rates demonstrated here can be attributed to the thicker chlamydospore wall seen in the average germinated chlamydospore in *P. ramorum* (1.6 µm), but more detailed experiments would have to be conducted to make a final determination. Other workers have reported chlamydospore germination rates of up to 60% in *P. ramorum*, but it is not clear how their results relate to those presented here.

It is unclear whether older chlamydospores of *P. ramorum* tend to germinate in greater numbers than younger chlamydospores. Chlamydospores that were grown in V8JB for 170, 90, and 10 days showed a significant difference in germination in older chlamydospores compared to younger by chi-square analysis. Although statistical analysis and percent germination indicated the germination rate to be higher in 170-day-old chlamydospores than either 90- or 10-day-old chlamydospores, it is not clear if this is a true effect or simply reflects the variation inherent in rates of chlamydospore germination in *P. ramorum*. In other experiments, 10-day-old V8JB chlamydospores treated in the same way, germinated at a rate that was greater (10.7 %) (Table 5) than the average germination rate of 170- (9.6 %), 90- (3.4 %), or 10- (2.2 %) day-old chlamydospores (Table 9). In other *Phytophthora* species age has either no effect (Mircetich *et al*. 1968; Holdaway & Tsao 1972) on chlamydospore germination or a negative effect (Chee 1973).

Germination of incubated chlamydospores takes place over at least five days on V8ARP media. Chlamydospores placed on V8ARP germinated at one, three, and five days after incubation on the media. This corresponded to very low overall germination rates of 0.1 % and 0.8 %. There was no further germination observed for up to 26 days. This result is in contrast to other workers who have recorded germination of *P. ramorum* chlamydospores stored on moist filters in sealed vials for 30 days at rates of 20 % and 50 % for chlamydospores stored in water (Davidson *et al*. 2002). One possibility for the differing results is that chlamydospores in the experiments reported here were left on the agar surface and the chlamydospores in Davidson's experiment were either stored on moist filters in sealed vials or in water. The agar surface could be a less aqueous environment than storage on a moist filter or in water and the chlamydospores dried out. Another possibility is that the presence of 20 µg/ml of Natamycin (a polyene antibiotic similar to Pimaricin) in the V8ARP inhibited chlamydospore germination or viability. A negative effect of Pimaricin has been recorded in other *Phytophthora* species. Ocana & Tsao (1965) recorded that chlamydospore germination of *P. cactorum*, *P. citrophthora*, and *P. parasitica* was inhibited at 100 µg of Pimaricin per ml but had no effect at 10 µg/ml. The presence of 20 µg/ml of Natamycin could have had a negative effect on chlamydospore germination, but the effect of Natamycin concentration has not yet been tested in *P. ramorum*. Non-empirical observation indicated that *P. ramorum* chlamydospores germinated at about the same rate in preliminary work done on V8ARP media; inhibitory effects of Natamycin were not overtly apparent.

The ability of the chlamydospore to germinate is central to its biological role in *Phytophthora*. It allows the fungus to establish new colonies and infect new hosts. It is therefore a vital aspect of chlamydospore biology to understand. There has been very little work done on chlamydospore germination in *P. ramorum*. The results presented here are the first time that consistently low and variable rates of chlamydospore germination in *P. ramorum* have been reported and the first time that germination has been examined in detail. This is the first report documenting evidence for the enhancement of chlamydospore germination with the presence of exogenous nutrients. This research also presents a first glimpse of the diameter and wall thickness of the average *P. ramorum* germinated chlamydospore. With an improved understanding of the factors that affect, inhibit, or enhance chlamydospore germination there is a better chance of developing mitigating measures to control future disease outbreaks. The results presented here on the germination of *P. ramorum* chlamydospores represent an important first new step towards this important goal.

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Chapter 4

Biology of Chlamydospores of *Phytophthora ramorum*: Chlamydospores in *Rhododendron* Leaves

Aaron Smith

Introduction

The chlamydospore is an important survival spore in *Phytophthora* that is readily formed in plant tissue in many *Phytophthora* species. Many of the chlamydospore-producing *Phytophthora* species are known to form chlamydospores in the roots of host plants, so it is vital that the chlamydospore is capable of withstanding the environmental and biotic pressures of the soil environment. Chlamydospore formation is less often recorded in stem and leaf tissue, but this too is known to occur frequently in some *Phytophthora* species. Chlamydospores formed in above-ground plant tissue are likely to encounter the same abiotic and biotic pressures as soil-borne chlamydospores because the dead host material, in which they have formed, will eventually be decomposed in a soil or aqueous environment. Chlamydospore formation within plant tissue is thought to be an important survival strategy for the fungus because it allows for added protection from desiccation, temperature extremes, and microbial antagonism over a longer period of time than it would otherwise encounter free from plant tissue in a non-sterile soil or aqueous environment. As plant tissue is decomposed over time, the chlamydospore is gradually exposed to an increasingly hostile environment in the soil, but it is also more likely to encounter a plant host. With this chance encounter and given the appropriate environmental conditions, the chlamydospore is able to germinate vegetatively or produce sporangia that subsequently release zoospores, which can infect host tissue directly. While in plant tissue, a new fungal colony is formed along with chlamydospores that will allow the fungus to survive after the current host is dead and until new host material is encountered some time in the future.

In-vitro studies provide an important first step toward understanding the parameters that affect chlamydospore formation, maturation, and germination, but it is the behavior of the chlamydospore in plant tissue that is most pertinent to gain an understanding of the biological role of the chlamydospore as it occurs in nature. In the tanoak forests of the quarantine zone of southwest Oregon a dominant understory plant is Pacific rhododendron. It is a known host of *P. ramorum* and a species that is capable of serving as a large source of inoculum in wildland ecosystems because of the ability of the fungus to produce a large number of chlamydospores in leaf and stem tissue (Tooley *et al*. 2004; Pogoda & Werres 2004). In southwest Oregon, understory Pacific rhododendron plants are known to become readily infected from rain drip from infected overstory tanoak trees (Goheen *et al.* 2002). These plants develop leaf and stem lesions and form chlamydospores within them (Goheen *et al*. 2002). What is not well understood is how the chlamydospore of *P. ramorum* behaves in Pacific rhododendron leaf tissue, after it is extracted, and what environmental conditions it is capable of surviving.

P. ramorum causes a necrosis on roots, stems, and leaves of Pacific rhododendron in wildland ecosystems of southwest Oregon and under greenhouse and detached leaf assay conditions. Chlamydospores are formed within the margins of necrotic tissue both on the exterior and interior of the

plant tissue (Tooley *et al*. 2004). Under greenhouse conditions *P. ramorum* has been shown to infect *Rhododendron* roots through soil infested with mycelium, that likely contained chlamydospores, and sporangia formed on vermiculite. Chlamydospores were formed in the cortex tissue of the roots (Lewis *et al.* 2004). Stems of Pacific rhododendron have been shown to develop a shoot dieback in wildland ecosystems of southwestern Oregon (Goheen *et al*. 2002) and under laboratory conditions (Pogoda & Werres 2004). Pogoda & Werres (2004) found, through histological analysis, that chlamydospores of *P. ramorum* only occurred in the brown twig zones where they developed primarily in the cortical parenchyma. Lewis (2005, *unpublished*) indicated that chlamydospores are also produced abundantly inside of detached *Rhododendron* leaves infected with *P. ramorum*.

Different hosts and cultivars produce varying numbers of chlamydospores in different parts of the plant. Davidson *et al.* (2002) reported chlamydospores forming on the surface of *Umbellularia californica* (California laurel) leaves, but not in the bark. Although chlamydospores have been reported in necrotic regions of stems and roots of *Rhododendron* plants, the numbers of chlamydospores produced in these tissues are relatively few compared to the number of chlamydospores reported in leaf lesions. Tooley *et al*. (2004) reported that the mean number of *P. ramorum* chlamydospores forming on the surfaces of host tissue of 21 different hosts ranged from 2 chlamydospores, in two different cultivars of *Vaccinium corymbosum* (highbush blueberry), to 921 chlamydospores on California laurel per 6-mm-diameter leaf disk. Among five different species of *Rhododendron*, four cultivars of *R. catawbiense* (Catawba rosebay), and one cultivar of *R. minus* (piedmont rhododendron), the number of chlamydospores produced ranged from 29 to 474 per 6-mm-diameter leaf disk (Tooley *et al*. 2004). There is also significant variation in the production of chlamydospores seen among cultivars of the same species. Tooley *et al*. (2004) reported that among five cultivars of *R. catawbiense* the numbers of chlamydospores produced ranged from 168 to 474 per 6-mm-diameter leaf disk. In resistant hosts, virtually no chlamydospores were produced indicating that host physiology affects the proficiency for chlamydospores to form in the tissue (Tooley *et al*. 2004).

P. ramorum produces thick-walled, large diameter chlamydospores in-vitro, but chlamydospores produced in stem and leaf tissue of *Rhododendron* are thicker and smaller in diameter. In a histological study of inoculated twigs of *Rhododendron*, Pogoda & Werres (2004) recorded *P. ramorum* chlamydospores in the cortical parenchyma with wall thicknesses ranging from about 3 to 7 µm and diameters of about 47 µm (based on visual estimates from the scale bar in their photomicrographs).

It is vital to the biological role of the chlamydospore to be able to germinate upon encountering the appropriate growth conditions and contact with a susceptible host. Chlamydospores of *P. ramorum* grown in *Rhododendron* leaves have been shown to germinate on media. Shishkoff & Tooley (2004) found that chlamydospores extracted from inoculated leaf tissue that was buried in mesh bags

germinated and formed colonies up to 155 days on selective media. Fichtner *et al*. (2005, *unpublished*) found that *P. ramorum*-inoculated *Rhododendron* leaves had recovery associated with chlamydospore germination of up to 80 % from leaves on the soil surface, 65 % from the soil and litter / soil interface. Inoculated *Rhododendron* and tanoak leaves that were both buried and kept at the soil surface for two to three months had up to 89 % and 50 % recovery on selective media respectively; this result was at least partially thought to be associated with chlamydospore germination (McLaughlin *et al*. 2005, *unpublished*).

Moisture strongly positively affects the survival and germination of *Phytophthora* chlamydospores. In many *Phytophthora* species, chlamydospores have survived for many years if they remain in a moist environment (Table 11). The ability of the chlamydospore to survive long periods in wet environments elucidates its biological role in *Phytophthora*. Water is also likely important in the germination of chlamydospores from plant tissue. Fichtner *et al*. (2005, *unpublished*) found that recovery of the fungus from infected *Rhododendron* leaves after eight weeks buried in the soil and at the litter and soil interface increased 3 to 10 % after incubation of the leaves in water for three weeks before returning the samples to media. The relationship of better recovery of the fungus with water treatment suggested the potential role of hydration in breaking chlamydospore dormancy, but this result could not solely be attributed to chlamydospores (Fichtner *et al*. 2005, *unpublished*).

Drying has been shown to have a negative effect on the survival of chlamydospores of *Phytophthora*. It has consistently been reported that no spore types (sporangia, zoospores, chlamydospores, or oospores) of different *Phytophthor*a species can survive more than a few days at matric potentials lower than about -0.1bar (Erwin *et al.* 1983), although there are conflicting reports related to chlamydospores. Air-drying soil from citrus groves in Florida reduces the recovery of *P. parasitica* propagules, composed primarily of chlamydospores and possibly some sporangia or mycelium, growing from germinated chlamydospores (Timmer *et al*. 1988). Sterne *et al*. (1977) demonstrated that as much as 70 % germination of *P. cinnamomi* chlamydospores could be obtained at matric potentials from 0 to -0.1 bars, whereas only 40-50 % germinated at -0.25 bars. Davidson *et al.* (2002) reported that *P. ramorum* chlamydospores were killed by a drying process of half an hour at 30 % relative humidity at 20 C in a dry filter treatment.

Chlamydospores of *Phytophthora* have differing tolerance to heat exposure. Chlamydospores of *P. cinnamomi* did not germinate from infested soil after exposure to 45 C for 20 minutes (Katan *et al*. 1976; Zentmyer *et al*. 1976). *P. nicotianae* failed to germinate after exposure to 53 C for only 3 minutes (Coelho *et al.* 2000). *P. ramorum* was found to be highly heat tolerant and was reisolated from inoculated California laurel leaves placed at 55 C for up to one week, but could not be recovered after two weeks at 55 C (Harnik *et al*. 2004). Linderman & Davis (2006) reported that chlamydospores of *P. ramorum* were killed in potting soil mix following an aerated steam treatment of 30 min. at 60 C.

Cold temperatures are thought to induce a winter dormancy in chlamydospores of most *Phytophthora* species in nature. Weste & Vithanage (1978) counted chlamydospores in soil samples from infested sites at intervals throughout two years using a soil sieving and plating technique that allowed chlamydospores to germinate on the media, embed their hyphae, and wash the soil off the plate with water leaving the germinated chlamydospores behind. The number of germinated chlamydospores increased to a maximum in the summer and autumn and very few chlamydospores were recovered during winter collections. A possible reason given for this result was that the chlamydospores entered into a dormant phase in the winter and dormant ungerminated chlamydospores would be washed off the plate (Weste & Vithanage 1978). In a later report Weste (1983) again found that during cool months, *P. cinnamomi* chlamydospores were undetectable in soil, but that populations increased rapidly after rain events. Lutz *et al*. (1991) documented the best known case of breaking winter dormancy. They positively correlated the germination of *P. parasitica* chlamydospores directly with the accumulation of heat units from 0 to 150 degree-days, but reported a negative correlation with germination with accumulation of heat units from 150 to 1650 degree-days with a maximum stimulation of 100-150 degree-days (Lutz *et al*. 1991). It is not known if winter dormancy occurs in the chlamydospores of *P. ramorum,* but the possibility has been suggested by Davidson *et al*. (2005).

Chlamydospores in plant tissue and soil environments must contend with microbial antagonism from fungi and bacteria. Fang (1995) documented the parasitism of *P. parasitica* chlamydospores by *Pythium nunn*. Chlamydospores were lysed by hyphal coiling and penetration. This interaction was species-specific in that *P. nunn* had no interaction with chlamydospores of *P. cinnamomi* or *P. citrophthora* (Fang 1995). Weste & Vithanage (1979) recorded the dynamic nature of *P. cinnamomi* chlamydospore populations in soil; chlamydospore populations were seen to increase in number in non-sterile, host-free soils, producing mycelium and more chlamydospores as long as there was a minimum of organic content in the soil substrate. Chlamydospores were negatively affected when the organic matter content was high and so too was the microbial competition and antagonism (Weste & Vithanage 1979). Chlamydospores of *P. colocasiae* that were produced in soil had a wall thickness of 1.8 µm or less, which was unlike thicker-walled chlamydospores (3.0 µm) produced in culture by Butler & Kulgarni (1913); the thinner-walled chlamydospores were more prone to lysis by soil microorganisms (Quitugua & Trujillo 1998).

Increased chlamydospore survival time in *Phytophthora* is closely tied to access to an aqueous or moist environment, reduced antagonism from soil microbes, and, to some degree, the thickness of the chlamydospore wall. In a literature review of recorded chlamydospore survival times in *Phytophthora*, several factors related to increased survival became apparent. Chlamydospore survival time was higher in sterile soil compared to non-sterile soil in *P. cactorum, P. citrophthora, P. parasitica*, and *P. sojae*; this result is likely due to the absence of antagonistic soil microorganisms (Table 11). Chlamydospore

survival was also higher in chlamydospores in roots compared to chlamydospores free in soil in *P. cinnamomi* and *P. lateralis* (Table 11); this result is likely due to the added protection provided by slowly decaying roots in soil from desiccation and antagonistic soil microorganisms. In an extraordinarily unique opportunity, Ko (2003) was able to test the survival of chlamydospores over six to 23 years. Under laboratory conditions chlamydospores of *P. cinnamomi, P. parasitica,* and *P. palmivora* each had their respective species' longest survival time stored in sterile distilled water at 20 C (Table 11). It is interesting to note that normally thin-walled (< 1.0 µm) species like *P. lateralis* and *P. citrophthora* (Chapter 2, Figure 3) are capable of surviving many years even in non-sterile soils (Table 11). Intermediate-walled species (1.0-1.5 µm) such as *P. cinnamomi* and *P. cactorum* (Chapter 2, Figure 3) are equally capable of surviving years in non-sterile soil (Table 11). Thick-walled *P. parasitica* (Average 2.2 µm) is capable of at least four years of survival in non-sterile soil and 18 years in sterile distilled water. Though long term tests over many years for the survival capabilities of thicker-walled chlamydospores *of P. ramorum* (Average 2.4 µm) have not yet been conducted, they are likely much longer than the current six months recorded based on a comparison of the chlamydospore survival times of other thinner-walled *Phytophthora* species (Table 11).

Table 11: Chlamydospore Survival Times of *Phytophthora* Species Under Different Environmental Conditions.

Chlamydospore Producing *Phytophthora* Species	Chlamydospore Survival Time	Survival Conditions	Reference
P. cactorum	3.5 months 2 years 6 years	Field soil Non-sterile soil Sterile soil	Gisi & Meyer (1973) Kröber (1980) Kröber (1980)
P. cinnamomi	6-8 months 30 days 100 days 1 year > 1 year 5 years 6 years 9-18 years	Gravel Field soil Buried roots -1/3 to -10 Bar Field soil Roots in soil Field soil Roots 20% moisture Sterile distilled water 20 C	Weste & Vithanage (1979) Weste (1983) MacKay *et al*. (1985) Hwang & Ko (1978) Hwang & Ko (1978) Weste (1975) Mircetich & Zentmyer (1966) Ko (2003)
P. citrophthora	3 years 4 years	Non-sterile soil Sterile soil	Kröber (1980) Kröber (1980)
P. lateralis	5 years 7 years	Soil bags Root systems in buried soil pot	Hansen & Hamm (1996) Hansen & Hamm (1996)
P. meadii	22 weeks	Field soil	Liyange & Wheeler (1991)
P. megasperma	7 months	Dried soil plates < 10 % moisture, 5-30 C	Basu (1980)
P. parasitica	30 days 60 days 180 days 4 years 6 years 6-18 years	Sterile soil Non-sterile soil Field soil 25% moisture, 24 C Non-sterile soil Sterile soil Sterile distilled water, 20 C	Ramarao & Umabala(1982) Ramarao & Umabala(1982) Holdaway & Tsao (1971) Kröber (1980) Kröber (1980) Ko (2003)
P. palmivora	19-23 years	Sterile distilled water, 20 C	Ko (2003)
P. ramorum	155 days 6 months 6 months	Buried leaves Potting mix Buried leaves	Shishkoff & Tooley (2004) Linderman & Davis (2006) Fichtner *et al*. (2005)
P. sojae	> 5 days < 5 days	Sterile soil Non-sterile soil	Ho (1969) Ho (1969)

The objectives of the experiments presented here were to better understand the chlamydospore of *P. ramorum* as it occurs in Pacific rhododendron leaves and make comparisons of chlamydospore development and germination as they occur in-vitro (i.e. Chapters 2 & 3) to the way they occur grown in leaves. This was accomplished by: (1) recording the rate of lesion development in detached *Rhododendron* leaves inoculated with *P. ramorum*; (2) measuring the number of chlamydospores produced in leaf lesions from two to 28 days of age; (3) measuring leaf-grown chlamydospore wall thickness and diameters and comparing those values with V8 agar and V8JB-grown chlamydospore

wall thickness and diameter (Chapter 2); (4) analyzing the composition of different chlamydospore wall thickness and diameter values found in leaf-grown chlamydospores and comparing those values with V8 agar and V8JB-grown chlamydospore wall thickness and diameter composition data (Chapter 2); (5) analyzing the relationship between chlamydospore diameter and wall thickness as it occurs in leaf-grown chlamydospores; (6) recording the germination of leaf-grown chlamydospores on V8 and cornmeal agars amended with antibiotics and antifungals (V8ARP and CARP) (Appendix B) over 12 days; and (7) recording the recovery of *P. ramorum* from leaf lesions of fresh, frozen, and air-dried leaves.

Methods

P. ramorum in Rhododendron macrophyllum Leaves

Leaf Lesion Growth: P. ramorum lesion development in *Rhododendron macrophyllum* D. Don ex G. Don leaves was measured in two experiments. *R. macrophyllum* leaves were collected from wild plants growing one mile west of Deadwood, OR on Highway 36. In experiment one, all leaves were rinsed with DIH_2O and nine randomly selected leaves were inoculated with a 5 mm^3 agar plug cut (Figure 27 Inoculated leaf) from the edge of an 11-day-old V8 agar culture of isolate 2027.1 by scoring the petiole and then securing the inoculum with moistened cotton and tin foil. In each replication, 12 leaves (nine inoculated and three negative controls) were suspended on wire mesh 30 mm above moistened paper towels on the bottom of a crisper and stored at 20 C in the dark. The experiment had four replications and was performed one time. Paper towels were moistened and leaf lesion growth was measured and outlined on the leaf surface with a permanent marker every two days for 28 days (Figure 27 Inoculated leaf).

In experiment two, *R. macrophyllum* leaves (same as above) and leaves from a domestic *R. macrophyllum* cultivar were inoculated by dipping the petiole ends in a zoospore suspension. The cultivar leaves were collected from the Oregon State University Campus, east side of Cordley Hall, Corvallis, OR. Leaves were inoculated at the petiole with zoospores from six 20-day-old V8 agar cultures of isolate P-110 each flooded with 5 ml DIH_2O and refrigerated for 30 minutes. Inoculated leaves, 12 wild type and ten cultivar, were placed directly on moistened paper towels in two separate crispers. Lesion boundaries were marked as in Experiment 1. Leaves were sampled three and seven days after inoculation and then every day for 27 and 33 days respectively. The experiment had no replications and was performed one time.

Leaf Clearing: Chlamydospores were counted in cleared leaf lesions from the leaf lesion growth experiment (Experiment 1). Half of the “middle” and “petiole” sections of three inoculated

(Figure 27 B) and uninoculated (negative control) leaves from each replication (Experiment 1) were placed in separate glass vials each containing 5.5 ml 10 % KOH (1g KOH: 10 ml DIH_2O) for five to seven days in an oven at 60 C, the KOH was replaced every 24 hours. The KOH was removed, samples were rinsed three times with DIH_2O, 5.5 ml of hydrogen peroxide was added for 30 minutes, and the samples were rinsed again three times with DIH_2O. In succession, 5.5 ml of 70 %, 80 %, 95 %, and 100 % EtOH, and 1:1 EtOH / Xylene was added for 10 minutes and removed, the samples were finally rinsed two times with 100 % Xylene and mounted on a microscope slide with Permount® (Fisher Scientific, SP15-100) and a cover slip.

Chlamydospores were counted in cleared leaf tissues using a Zeiss compound microscope and phase contrast illumination. Chlamydospores were counted in three 200x magnification FOV in one tissue piece from each lesion age class from both the "middle" and the "petiole" portions of the leaves (Figure 27 B) for inoculated and uninoculated leaves. Only the tissue between leaf veins was examined, by focusing on stomates on the leaf surface and then slowly focusing up and down through the depth of the leaf tissue. Chlamydospores appeared as thick-walled "light rings" and were tallied if any portion of a chlamydospore was within the FOV; questionable objects were not tallied.

The number of chlamydospores in leaf lesions was calculated. Leaf lesion area was calculated as the area of two right triangles (Figure 27 C) with the heights equal to the average of 36 lesion lengths and the base equal to average lateral growth of two to 15 lesions in the age classes zero to 16 days old from "middle" sections of leaf lesions (Figure 27 B). The average area of each age class was calculated by taking the average height and width growth increment, and subtracting the age class area from the total area of the lesion (Figure 27 C). In age classes two to 16 days old the number of chlamydospores in the area of each age class was calculated by multiplying the mean number of chlamydospores per 200x FOV (1.02 mm^2) by the number of FOV that cover the area of each age class (data not shown). In age classes 18 to 28 the mean number of chlamydospores was averaged across all age classes.

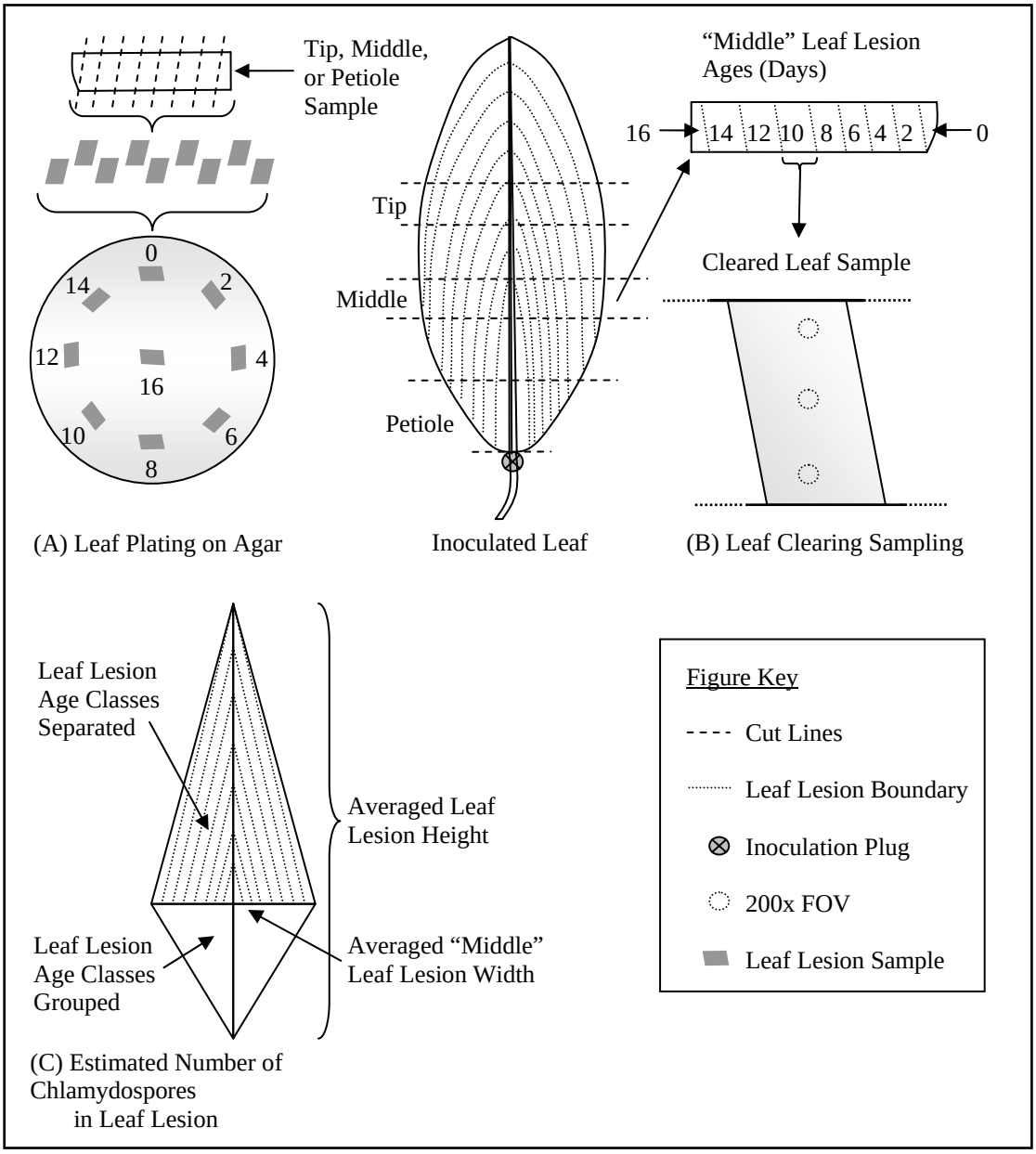
Diameter and Wall Thickness of Leaf-grown Chlamydospores: Diameter and wall thickness of 28-day-old chlamydospores from the germination of leaf-grown chlamydospores was measured. 50 chlamydospores were measured after an additional 14 days on CARP and 50 were measured after being frozen for 15 days and on V8ARP for seven days. Chlamydospores were measured on 150 mm diameter agar plugs with a cover slip at 1000 x magnification under bright field illumination. The samples were determined to not be significantly different from each other with 95% confidence interval analysis and were combined to represent leaf-grown chlamydospores. Leaf-grown chlamydospore wall thicknesses and diameters were compared to V8 agar and V8JB grown wall thicknesses and diameters (The same chlamydospores from Chapter 2, Chlamydospore Maturation on V8 Agar and V8JB Experiments).

Germination of Leaf-grown Chlamydospores Over Time: Germination of chlamydospores extracted from leaves (leaf lesion growth Experiment 1) was compared on V8 and cornmeal agar amended with antibiotics (Appendix B: V8ARP and CARP). Chlamydospores were extracted by pulverizing 1 mm leaf strips from 24 leaf lesion areas not outlined as "tip", "middle", or "petiole" (Figure 27 Inoculated Leaf) with ten three-mm-glass beads and one ml DIH_2O for one minute in a bead beater (Biospec Products, Bartlesville, OK, USA). Pulverized tissue was rinsed through a sieve, the rinsate was collected, transferred to filters, and then to eight Petri dishes as described in Chapter 3 (Separation of Chlamydospores, Figure 21). Petri dishes were checked for chlamydospore germination every two days for 12 days by eye and compound microscope at 100x magnification.

Effect of Freezing and Air-Drying on P. ramorum Survival: Effects of freezing and air-drying on fungus survival in leaf lesions was analyzed using tissue from the leaf lesion growth experiment (Experiment 1). One half of the "tip", "middle", and "petiole" sections from 12 uninoculated control and 12 inoculated fresh leaves (Figure 27 Inoculated Leaf) were cut into age classes and plated on CARP (Figure 27 A), sealed with Parafilm®, and checked for growth of the fungus after three days by compound microscope at 100x magnification. The three complete sections (Figure 27 Inoculated Leaf) from 24 leaves were placed in paper envelopes and frozen (12 leaves) or air-dried at 20 C in the dark (12 leaves) for 5, 10, or 15 days. After treatment all three sections from three leaves of each treatment were cut into age classes (Figure 27 A), placed on CARP in Petri dishes, sealed with Parafilm®, and checked for growth five and ten days after plating. Chlamydospores were extracted from the leaf pieces frozen for 15 days and germination was attempted on V8ARP, as described above. After 15 days of treatment, germination of chlamydospores was attempted following the procedure in leaf-grown chlamydospore germination, except that the chlamydospores were hydrated in water for 24 hours; Petri dishes were checked for germination each day for six days.

Statistical Analysis of Leaf-grown Chlamydospores: Mean lesion growth (MLG), mean number of chlamydospores per 200x FOV, and mean chlamydospore wall thickness and diameter were calculated using S-plus® 7.0 statistical software, by calculating the mean of the measured values. The associated range of values were constructed with minimum and maximum values. The relationship between chlamydospore diameter and wall thickness was calculated by adding a trend line with its associated r^2 value and formula to a scatter plot of wall thickness and diameter values recorded in the leaf-grown chlamydospore sample. Graphs were created with Microsoft® Excel 2002 and S-plus® 7.0.

Figure 27: Experimental Design Graphic for Experiments Related to *Rhododendron* Leaf Inoculation.



Results

P. ramorum in Rhododendron macrophyllum Leaves

R. macrophyllum leaves inoculated with *P. ramorum* developed black lesions in the midrib, veins, and fleshy tissue all of which contained chlamydospores within three days of becoming necrotic. Chlamydospores that developed in leaf tissue were spherical, ovoid, and very often irregular. Mature chlamydospores extracted from lesions were dark brown in bright field illumination on an agar surface.

Leaf Lesion Growth: Necrotic lesions in inoculated *R. macrophyllum* leaves expanded 4.5 and 7 mm/day in experiments one and two respectively, and 3.1 mm / day in *Rhododendron* cultivar leaves (Table 12).

Table 12: Mean Lesion Growth (MLG) in *Rhododendron macrophyllum* Leaves Inoculated with *P. ramorum*.

Exp.	Leaf Type	All Replications MLG (Range) (mm/day)	Replication 1 MLG (Range) (mm/day)	Replication 2 MLG (Range) (mm/day)	Replication 3 MLG (Range) (mm/day)	Replication 4 MLG (Range) (mm/day)
One	Wild type	4.5 (0.3-14.5)	4.7 (0.8-10.8)	4.8 (0.5-14.5)	4.4 (0.3-14.0)	4.2 (0.8-12.0)
Two	Wild type	7.2 (0-40.0)				
	Cultivar	3.1 (0-8.0)				

Leaf Clearing: The mean number of chlamydospores per 1.0 mm^2 FOV in leaf lesions (excluding leaf veins) increased from 0.5 in the two-day-old leaf lesion age class to 22.8 in the 16-day-old leaf lesion age class with some variation (Table 13). The mean number of chlamydospores counted decreased significantly in the 18- to 28-day-old leaf lesion age classes to an average of 8.4 (average excluding value for replication 2 due to a possible error in the value) in all age classes and the numbers were more variable than younger age classes (Table 13). The estimated total number of chlamydospores in an average 28-day-old leaf lesion with a total length of 105 mm and width of 38.6 mm was about 10,000.

Table 13: Mean and Number of Chlamydospores per 200x FOV (1.0 mm^2) in *R. macrophyllum* Leaves Inoculated with *P. ramorum*, Incubated 2-28 Days, and Cleared with KOH.

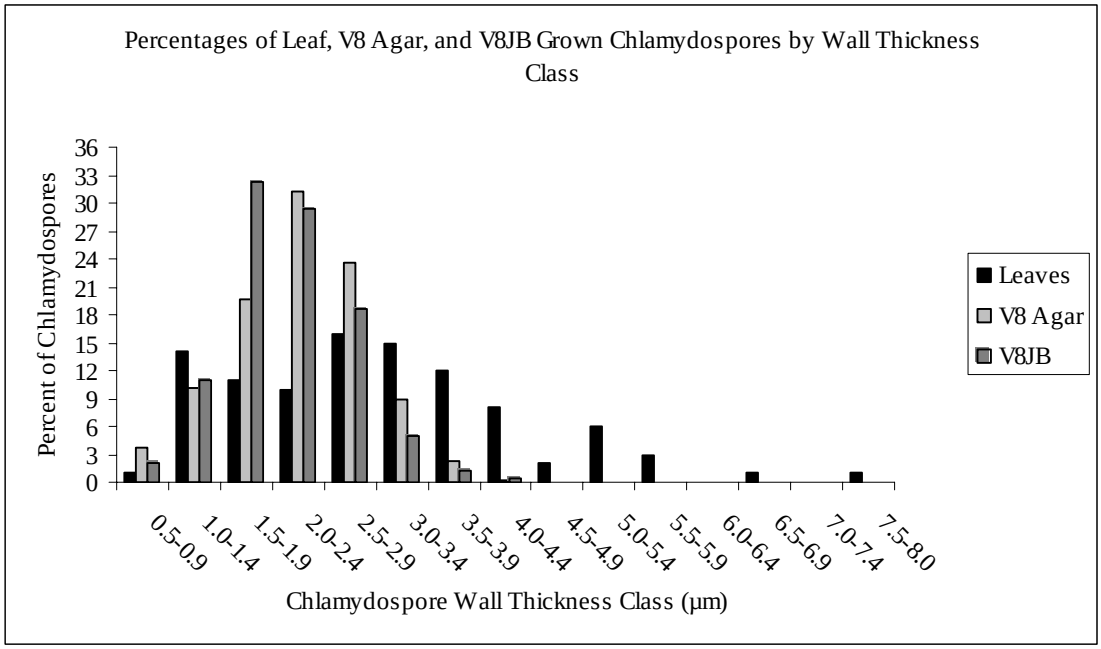
Leaf Age Class (Day)	All Replications Mean Number of Chlamydospores / 200x FOV (Range)	Replication 1 Number of Chlamydospore	Replication 2 Number of Chlamydospore	Replication 3 Number of Chlamydospore	Replication 4 Number of Chlamydospore
2	0.5 (0-2.0)	0	0	0	2.0
4	3.8 (0-12.0)	2.0	12.0	0	1.0
6	7.5 (2.0-20.0)	5.0	20.0	3.0	2.0
8	15.3 (8.0-30.0)	9.0	14.0	30.0	8.0
10	24.0 (18.0-31.0)	18.0	19.0	31.0	28.0
12	25.5 (21.0-29.0)	29.0	21.0	29.0	23.0
14	20.3 (0-40.0)	0	26.0	40.0	15.0
16	22.8 (0-45.0)	0	45.0	31.0	15.0
18-28	8.4 (6.0-163.0)	6.0	163.0	20.0	13.0

Diameter and Wall Thickness of Leaf-grown Chlamydospores: Leaf-grown chlamydospore wall thickness was thicker (2.9 µm) than V8 agar (2.1 µm)- or V8JB (2.1 µm)- grown chlamydospores in two- to 30-day-old chlamydospores (Table 14). Leaf-grown chlamydospores had maximum wall thicknesses greater (5.0-8.0 µm) than V8 agar (4.75 µm)- or V8JB (4.25 µm)-grown chlamydospores (Figure 28).

Table 14: Comparison of Wall Thickness in Leaf-, V8 agar-, and V8JB-grown Chlamydospores

Treatment	Mean Chlamydospore Wall Thickness (µm) (Range)
Leaf-grown Chlamydospores	2.9 (0.8-8.0)
V8 Agar-grown Chlamydospores	2.1 (0.5-4.8)
V8JB-grown Chlamydospores	2.1 (0.6-4.3)

Figure 28: Percentages of Leaf-, V8 Agar-, and V8JB-grown Chlamydospores by Wall Thickness Class

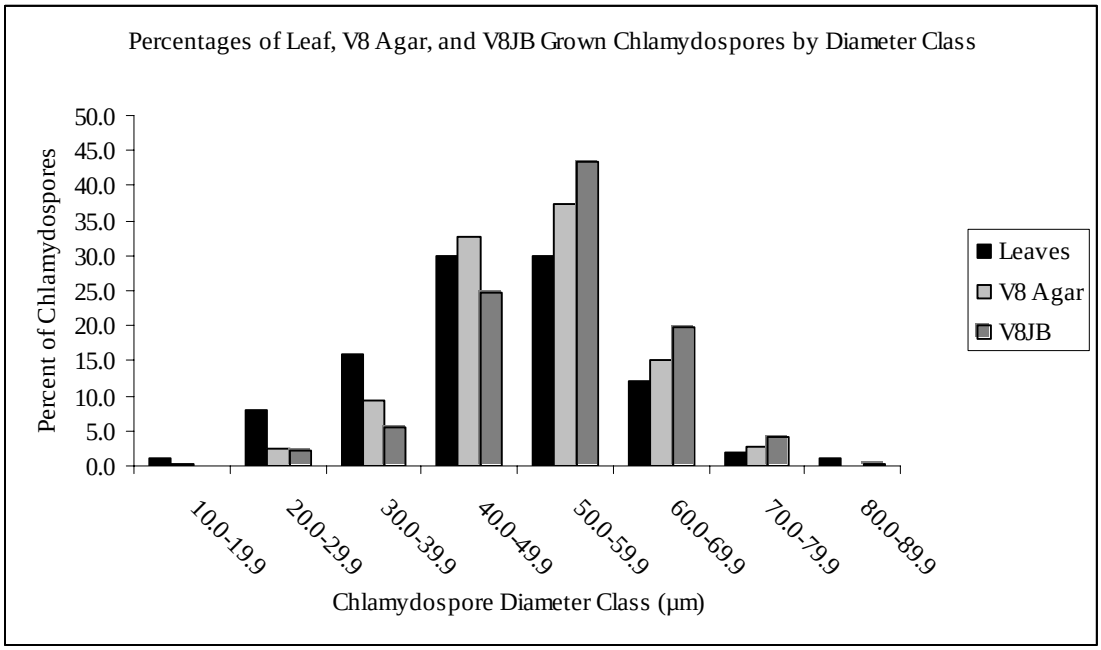


Leaf-grown chlamydospore diameter was smaller (47.6 µm) than V8 agar (50.8 µm)- or V8JB (54.0 µm)-grown chlamydospores in two- to 30-day-old chlamydospores (Table 15). Greater percentages of leaf-grown chlamydospores were represented in the smaller diameter classes and smaller percentages were represented in the larger diameter classes than in V8 agar or V8JB (Figure 29).

Table 15: Comparison of Leaf-, V8 Agar-, and V8JB-grown Mean Chlamydospore Diameter

Treatment	Mean Chlamydospore Diameter (µm) (Range)
Leaf-grown Chlamydospores	47.6 (19.0-80.0)
V8 Agar-grown Chlamydospores	50.8 (15.5-80.5)
V8JB-grown Chlamydospores	54.0 (21.0-81.8)

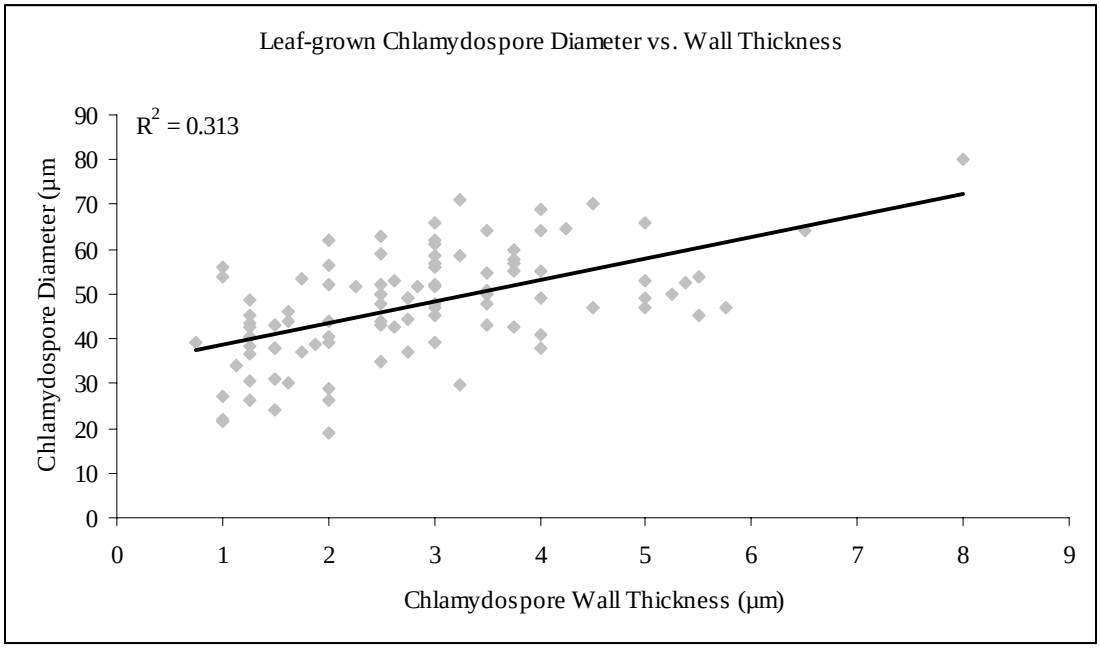
Figure 29: Percentages of Leaf-, V8 Agar-, and V8JB-grown Chlamydospores by Diameter Class



Relationship Between Leaf-grown Chlamydospore Diameter and Wall Thickness:

Chlamydospore diameter increased with wall thickness in leaf-grown chlamydospores aged two- to 28-days-old (Figure 30). The r^2 value for two- to 28-day-old leaf-grown chlamydospores was less (0.31) (Figure 30) than V8 agar (0.47) or 10- to 120-day-old V8JB (0.37) chlamydospores (Chapter 2, Figures 18 & 19).

Figure 30: Relationship Between Diameter and Wall Thickness in *Rhododendron* Leaf-grown Chlamydospores



Germination of Leaf-grown Chlamydospores Over Time: Fresh leaf-grown chlamydospores extracted from lesions 0-28 days old, germinated in higher numbers when plated on V8ARP (22) than on CARP (1). On V8ARP, germination continued for at least 12 days (Table 16).

Table 16: Germination of Leaf-grown Chlamydospores Over Time

Number of Days Since Plating on Agar Media	Number of Chlamydospores Germinated on V8ARP	Number of Chlamydospores Germinated on CARP
0	0	0
2	5.0	0
4	7.0	1.0
6	7.0	0
8	1.0	0
10	1.0	0
12	1.0	0
Totals	22.0	1.0

P. ramorum Survival after Freezing and Drying: P. ramorum was recovered from 100 % of samples from fresh lesions of all age classes from inoculated leaves (Table 17). No *P. ramorum* was recovered from uninoculated control samples, but other fungi were isolated. All inoculated and uninoculated control frozen and air-dried lesion samples were negative for *P. ramorum* at all sample times (Table 17). Chlamydospores extracted from leaf lesions frozen 15 days showed no germination after six days on V8ARP (Table 18).

Table 17: *P. ramorum* Recovery from *Rhododendron* Leaf Lesions

Treatment	Number of Days Since Plating on CARP	*Phytophthora ramorum* Present (number positive samples / number of samples)
Inoculated Fresh	3	36 / 36
Uninoculated Fresh	6	0 / 36
Inoculated Frozen 5 Days	5	0 / 12
	10	0 / 12
Inoculated Dried 5 Days	5	0 / 12
	10	0 / 12
Inoculated Frozen 10 Days	6	0 / 12
	10	0 / 12
Inoculated Dried 10 Days	6	0 / 12
	10	0 / 12

Table 18: Germination of Extracted Leaf-grown Chlamydospores after 15 Days of Freezing and Germinated on V8ARP

Leaf Tissue Prepared for Chlamydospore Germination	Number of Days Since Plating on V8ARP	*P. ramorum* Germination
Inoculated Frozen 15 Days	6	None

Discussion

P. ramorum had an average advancing lesion development rate of 4.5 (isolate 2027.1) and 7.2 (isolate P-110) mm/ day along the midrib of detached Pacific rhododendron leaves. Lesion expansion developed at less than half the rate (3.1 mm/day) of inoculated Pacific rhododendron leaves in a *Rhododendron* cultivar inoculated with isolate P-110. Chlamydospores are abundantly formed inside the leaf tissue within the boundaries of necrotic lesions. A larger number of chlamydospores are found in older lesion tissue than in younger lesion tissue. The average number of chlamydospores formed ranged from 0.5 to 22.8 chlamydospores per 1.0 mm^2 in lesions age classes two to 16 days old respectively. The total number of chlamydospores formed in a 28-day-old *Rhododendron* leaf lesion was estimated to be around 10,000. *Rhododendron* leaf-grown chlamydospores form a thicker mean wall (2.9 µm) than V8 agar- or V8JB (2.1µm)-grown chlamydospores. Leaf-grown chlamydospores also tend to become much thicker (5.0 to 8.0 µm) than chlamydospores grown in V8 agar (4.8 µm) or V8JB (4.3 µm). Leaf-grown chlamydospores tend to have a smaller mean diameter (47.6 µm) than V8 agar (50.8 µm) or V8JB (54.0 µm) grown chlamydospores. There is a strong relationship between chlamydospore diameter and wall thickness in leaf-, V8 agar-, and V8JB-grown chlamydospores; chlamydospore wall thickness tends to increase with chlamydospore diameter. Leaf-grown chlamydospores extracted from leaf tissue germinate at a low frequency over 12 days on V8 agar amended with antibiotics (V8ARP). *P. ramorum* was recovered from 100 % of fresh inoculated leaf

lesion tissue on cornmeal agar amended with antibiotics (CARP). Leaf lesions frozen or air-dried for 5 or 10 days resulted in no recovery of *P. ramorum* on CARP. Chlamydospores extracted from leaf lesions frozen for 15 days also failed to germinate over six days on V8ARP.

Different isolates of *P. ramorum* form lesions in detached *Rhododendron* leaves at different rates. Isolate 2027.1 (an Oregon forest isolate) had an average lesion development rate of 4.4 mm/ day along the midrib of pacific *Rhododendron* leaves. Average lesion development was 7.2 mm/ day for isolate P-110 (a California isolate), significantly faster. The leaves used in the experiment were taken from the same plant, collected two years apart in February. Differences in plant physiology related to a plant-level response to differing environmental conditions present in each respective year may account for the differences in lesion development rates of *P. ramorum*, although it would be surprising if this accounted for all of the variation. Further tests with these isolates will be required to determine the extent of isolate growth variation in *Rhododendron* leaves. Growth rate variation has been recorded among different isolates of *P. ramorum*, although only in in-vitro studies. Werres *et al.* (2001) found that growth rates of 14 *P. ramorum* isolates from the Netherlands and Germany varied between 2.5 to 3.5 mm/ day on carrot piece agar (CPA) at each isolate's optimum growth rate temperature. It is interesting to note that for both isolates, 2027.1 and P-110, the growth rate was faster in detached *Rhododendron* leaves than growth recorded under optimum in-vitro conditions on a highly nutritive agar among European isolates. Using one of the isolates (BBA 9/95) Pogoda & Werres (2004) performed cut-stem inoculations of *Rhododendron* cv. 'Cunningham's White'. The average stem lesion development was 6.5 mm/ day over 12 days (Pogoda & Werres 2004), whereas the optimum growth rate of BBA 9/95 was less than half as fast at 2.8 mm/ day on CPA (Werres *et al.* 2001).

Chlamydospores are abundantly formed within the necrotic margins of *P. ramorum* inoculated *Rhododendron* lesions in detached leaves. Leaf clearings of detached leaf *Rhododendron* lesions indicated that chlamydospores only form within the necrotic margins of leaf lesions; no chlamydospores were seen in the green tissue outside of the necrotic margin. Surfaces of leaves were not checked for the presence of chlamydospores. The recovery of the fungus from leaf lesion platings on CARP indicated that the fungus was present in the green tissue, likely in the form of hyphae, but at a relatively lower incidence compared to plated necrotic tissue. The formation of chlamydospores within the lesion material was found to vary depending on the amount of time since the lesion tissue had become necrotic (i.e. age classes). The mean number of chlamydospores formed increased from 0.5 to 22.8 chlamydospores per 1.0 mm^2 in the lesion age classes between two and 16 days (Table 13). Chlamydospores begin forming in necrotic portions of the lesion starting at 2 days old; as the lesion ages, more chlamydospores are formed in the portions of the lesion that have been infected longer. *P. ramorum* growing on V8 agar and V8JB formed chlamydospores within two to three days (Chapter 2),

a result that is in congruence with the chlamydospore incidence gradient seen in *Rhododendron* leaf lesions.

An interesting result from the leaf clearing was that in older lesion sections, the mean number of chlamydospores dropped dramatically from 22.8 / 1.0 mm^2 in 16-day-old lesions to an average of only 8.4 chlamydospores / 1.0 mm^2 in lesions aged 18-28 days. There are a couple possible explanations for this result: (1) After 28 days in the moist, humid environment of the crisper box, some degree of fungal growth from other unidentified species occurred on the surface of the leaves. Endophytic or surface fungi from the leaves could have colonized necrotic leaf tissue and had an antagonistic effect on chlamydospores already formed in lesion material in the incipient stages of decay. In platings of leaf tissue frozen and dried for 5 or 10 days, a number of unidentified fungal species were seen to grow out of the leaves on CARP. Although this strongly indicates the presence of other fungi within the leaf tissue along with chlamydospores of *P. ramorum,* it is not, in itself, indicative of microbial antagonism. (2) In the inoculated detached leaves used in the experiment, the measurement of the advancement of the leaf lesion was recorded every two days with a permanent marker. The ink from the marker was not completely faded from the leaf surface by the leaf clearing process in the portions of the lesion 18-28 days old. This obscured the view through the leaf tissue under phase contrast illumination in many sections of the older portions of leaf lesion and could have led to a reduced number of chlamydospores tallied in these sections. This is likely the reason for the reduced number of chlamydospores sampled in older portions of the leaf lesion, but it remains unclear at this writing.

The total number of chlamydospores that are formed within *P. ramorum* infected Pacific rhododendron leaves is surprisingly high. A calculation of the total number of chlamydospores that are formed within a typical leaf lesion 2,024 mm^2 (dimensions = 105 x 38.6 mm, n = 15-36 lesions) was estimated to be around 10,000 +/- 7450 chlamydospores. There were several important assumptions made in this calculation: (1) The lesion age classes 2-16 were assumed to be trapezoidal areas (Figure 27 C) for the purposes of simplifying the calculation; in reality they were curved and somewhat irregular (Figure 27 Inoculated Leaf). (2) The lesion age classes 18-28 were grouped together and the number of chlamydospores recorded for those areas were averaged and taken to be representative of the number of chlamydospores that could be expected to form in those age classes. This was due to the difficulty of distinguishing between two days growth delineated on the leaf surface after 28 days of overlapping ink marks. (3) It was assumed that all portions of the individual age classes had as many chlamydospores as were recorded in the sampling; in fact, only two sections of these age classes were sampled in the 2-16 age classes and one in the 18-28 age classes (Figure 27 Inoculated Leaf) (4) It was assumed that half of the total leaf lesion area of 2,024 mm^2 was composed of vein tissue, which was not sampled for presence of chlamydospores in this analysis; this area was not included in the calculation.

The area of vein tissue was only taken as an observational estimate and was not validated empirically. (5) No chlamydospores that formed in the midrib of the leaf lesion were recorded, though preliminary analysis indicated that chlamydospores are formed in the midrib. This calculation only serves as a rough estimate of the number of chlamydospores than can be expected to form in *Rhododendron* leaf lesions. It is, however, striking that even the lowest estimated number (2550 chlamydospores) is alarmingly high for a single leaf and is, in itself, illustrative of the impressive inoculum potential of infected Pacific rhododendron plants in a wildland ecosystem of southwestern Oregon or in a nursery setting.

There is good supporting evidence from other workers that indicate the estimated number of chlamydospores that can be expected to form in *Rhododendron* leaves infected with *P. ramorum* and recorded here is highly probable. Tooley *et al.* (2004) inoculated leaf disks of five different species of *Rhododendron* along with five cultivars of two of the species, with an A2 isolate from California. After 12 days of incubation at 20 C, they recorded the number of chlamydospores formed on the abaxial and adaxial surfaces of the disks, not in the interior tissue. Tooley *et al.* recorded chlamydospore formation ranging from 1 to 17 chlamydospores / mm^2 across all species and cultivars. Their highest value of 17 chlamydospores / mm^2 was recorded on *Rhododendron catawbiense* cv. 'Cunningham's White', unfortunately Pacific rhododendron was not tested. The mean number of chlamydospores formed in 12-day-old lesions in this thesis indicate the formation of 26 (range 21-29) chlamydospores / mm^2 (Table 13). Although Tooley *et al.* only recorded chlamydospores on the surfaces of leaf discs, the similarities of the two measurements would suggest that the number of chlamydospores seen in 12 day-old leaf lesions from this work is very close to Tooley *et al*. (2004) published values.

P. ramorum chlamydospores grown in *Rhododendron* leaf tissue are thicker-walled than chlamydospores grown in V8 agar or V8JB. Mean chlamydospore wall thickness in two- to 30-day-old chlamydospores was 2.9 µm for leaf-grown and 2.1 µm for V8 agar and V8JB chlamydospores (Table 14). Leaf-grown chlamydospores also tended to get thicker than V8- or V8JB-grown chlamydospores; wall thickness ranged from 5.0 to 8.0 µm in leaf-grown and was 4.8 µm and 4.3 µm in V8- and V8JB-grown chlamydospores respectively (Figure 28). Thicker chlamydospore walls forming in plant tissue has also been reported by Pogoda & Werres (2004) who found that twigs of *Rhododendron* inoculated with *P. ramorum* had chlamydospores in the cortical parenchyma with wall thicknesses ranging from about 3 to 7 µm (based on visual estimates from the scale bar in their photomicrographs). Chlamydospores in other *Phytophthora* species exhibit this behavior as well. Observations of naturally infected papaya fruits revealed that as many as 90 % of the chlamydospores produced by *P. palmivora* were thick-walled (>1.5 µm) (Kadooka & Ko 1973). More than 70 % of the chlamydospores produced by *P. palmivora* grown in a papaya juice medium were thick-walled and were similar to those obtained in nature (Kadooka & Ko 1973). Only thin-walled (<1.0 µm) chlamydospores were produced in 20 %

V8JB culture and stored submerged in water (Tsao 1971; Kadooka & Ko 1973). Chlamydospores of *P. capsici* grown in 10 % submerged V8JB produced numerous chlamydospores with walls 1.0-1.5 µm thick. Thicker-walled (2.0 µm) chlamydospores of *P. capsici* were produced in cultures grown on autoclaved pepper fruit (Uchida & Aragaki 1985). Shew & Benson (1982) observed that many of the chlamydospores of *P. cinnamomi* that formed naturally in soil had thick walls (mean thickness, 1.75 µm). They noted that chlamydospores that are produced in culture do not typically have a thicker inner wall, but chlamydospores produced in nature do. The thicker wall was thought to enhance long-term survival of *P. cinnamomi* in soil, and/ or may be a response to substrate or to biotic stresses present in soil (Shew & Benson 1982). The thicker wall found in chlamydospores grown in natural environments such as plant tissue or in soil substrates, is a common phenomenon in *Phytophthora* species. It is likely triggered by a difference in nutrients present in plant material, a difference in environmental conditions, a biological response to microbial antagonism, or a combination of these factors, that the fungus does not encounter in culture (Kadooka & Ko 1973). The formation of a thicker chlamydospore wall is likely a survival strategy to allow the chlamydospore to better survive extremes in temperature, lack of moisture, and microbial antagonism.

Rhododendron leaf-grown chlamydospores of *P. ramorum* tended to be smaller in diameter than chlamydospores grown in culture. Leaf-grown chlamydospores were smaller in diameter at 47.6 µm compared to V8 agar (50.8 µm) and V8JB-grown (54.0 µm) chlamydospores (Table 15). Pogoda & Werres (2004) also documented smaller (47 µm) diameter *P. ramorum* chlamydospores from lesions in twigs of *Rhododendron* (based on visual estimates from the scale bar in their photomicrographs). It is not clear why chlamydospores formed within plant tissue are smaller, but several possibilities exist. (1) Chlamydospores are formed within the confined spaces of the cellular structure and vein tissue within the plant. Observations from this thesis indicate that chlamydospores of *P. ramorum* are more frequently irregular rather than spherical when formed in leaf tissue, presumably because of the influence of the cellular structure within the leaf during chlamydospore development. This could restrict the final size of the chlamydospore. (2) It is possible that chlamydospore diameter is influenced by the vigor and size of the parent culture in which they are produced. Clearly cultures of *P. ramorum* growing in V8 agar or V8JB have developed far more interconnected hyphae than cultures that are growing in *Rhododendron* leaves. Therefore, agar and juice broth cultures are likely to be taking up more nutrients from the media and producing more cytoplasm. Cytoplasm is thought to actively expand the thin chlamydospore expansion wall through hydraulic pressure (Bartnicki-Garcia & Hemmes 1975); it stands to reason that cultures with more hyphae and unlimited access to nutrients should produce larger chlamydospores. In contrast, chlamydospores produced in leaves likely have access to far less cytoplasm with which to expand their walls. There have been no experiments conducted in *Phytophthora* chlamydospores to adequately describe this phenomenon.

Chlamydospores of *P. ramorum* grown in leaves exhibit a relationship between diameter and wall thickness; chlamydospore wall thickness increases with diameter. This relationship is seen in leaf-, V8 agar-, and V8JB-grown chlamydospores up to 28, 31, and 120 days old respectively. The r^2 value was less in leaf-grown chlamydospores (0.31) than in either V8 agar (0.47) or V8JB (0.37) (Figure 30, Chapter 2, Figures 18 & 19), indicating a weaker relationship in leaf-grown chlamydospores. A likely contributor to this result is that the sample size for leaf-grown chlamydospores was much smaller (n = 100) compared to V8 agar (n =2100) or V8JB (n =900). The strongest relationship (0.47 for V8 agar) is seen in the largest sample size, the weakest in the smallest (0.31 for leaves) sample size. It is very likely that there is no true difference in the strength of the relationship between chlamydospores grown in the three different media as the results of these analyses would indicate.

Phytophthora chlamydospores grown in plant tissue are morphologically and physiologically different than chlamydospores grown in culture. Kadooka & Ko (1973) documented these differences in papaya-grown chlamydospores of *P. palmivora*.

> Besides the morphological difference, thick-walled chlamydospore differed from thin-walled chlamydospores in that (i) thick-walled chlamydospores germinated poorly [V8 agar 5 %, Noble water agar 1 %, Water 17 %], but the thin-walled chlamydospore germination was nearly 100 % in distilled water, on V8 juice agar and Noble water agar; and (ii) thick-walled chlamydospores had a higher tolerance to high temperature than thin-walled chlamydospores [After exposure to 46 C for 5 min., 15 % thin-walled (V8JB-grown) chlamydospores germinated on Bacto water agar whereas about 85 % of thick-walled (papaya fruit-grown) germinated]. This indicates that chlamydospores produced by some artificial methods differ both morphologically and physiologically from those produced in nature (Kadooka & Ko 1973). Information in brackets was added by the author for clarification.

In this thesis, extracted *P. ramorum* chlamydospores grown for 28 days in *Rhododendron* leaves germinated at low rates on V8 agar amended with antibiotics (V8ARP). Chlamydospore germination occurred over 12 days, but frequency of germination was unevenly distributed over that time. The number of germinated chlamydospores was five after two days of incubation at 20 C on agar, increased to seven after four and six days, and dropped to one chlamydospore every other day up to 12 days (Table 16). It is interesting to note that in a separate chlamydospore germination (Chapter 3, Table 10) of chlamydospores grown for 10 days in V8JB, that germination was even lower and germination ceased in a shorter period of time on V8ARP. The number of germinated chlamydospores was two and one after one and five days respectively in the first experiment and five and four after one and three days respectively in the second experiment. This rate of germination corresponded to about 0.1 and 0.8 % germination for V8JB-grown chlamydospores in experiment one and two respectively. In both the leaf-grown and V8JB-grown germinations, chlamydospore germination dropped off dramatically after five to six days on the agar surface; dropping to a rate of a single chlamydospore germinating every other day for leaf-grown and no germination for V8JB-grown. It is possible that this reduction in

chlamydospore germination threshold is caused by a slow drying of the chlamydospore on the agar surface over time, eventually forcing it into dormancy or killing it. Davidson *et al.* (2002) reported that *P. ramorum* chlamydospores were killed by a drying process of half an hour at 30 % relative humidity at 20 C in a dry filter treatment. Even though the matrix of the agar is itself a moist environment (about 99 % water), only a small portion of the chlamydospore is in contact with the agar surface; the majority is exposed to air and therefore more susceptible to the negative effects of drying. The agar itself becomes drier over time as well. More experiments are needed to better understand this relationship. It is interesting, however, that the thicker-walled leaf-grown chlamydospores (Average 2.9 µm) were able to continue to germinate over at least twice as many days as thinner-walled V8JB-grown chlamydospores (Ten-day-old average 1.8µm [Chapter 2, Table 3]) on a V8ARP agar surface. More detailed experiments with leaf-grown and V8JB-grown chlamydospores need to be conducted to better understand this result. The morphological and physiological differences found in plant-grown chlamydospores highlights the importance of further in-planta tests to better understand the behavior of chlamydospores from plant tissue.

P. ramorum-infected *Rhododendron* leaf lesions have a very high rate of recovery of the fungus when plated fresh on CARP, but exhibit a drastic reduction in recovery of the fungus when subjected to freezing temperatures or air-drying. In 28-day-old leaf-lesions, the rate of recovery of fresh platings was 100 % (36 positives out of 36 samples) uninoculated controls had no recovery (Table 17). When the leaf lesions were subjected to freezing temperatures or air dried for 5 or 10 days, there was no recovery of the fungus on CARP for up to 10 days (Table 17). It is important to note that it is only likely that any recovery of the fungus from leaf lesions exposed to freezing or drying would be due to chlamydospore survival because they are likely more resistant to these conditions than sporangia or hyphae. It does not, however, exclude the possibility that that sporangia or hyphae would also survive this treatment and be recovered on agar. The negative effects of drying on chlamydospore survival was partially corroborated by Davidson *et al.* (2002) who reported death of chlamydospores after only 30 minutes of air-drying. In stark contrast to the results presented here, Rizzo (*unpublished*) reported that detached *Rhododendron* leaves that were dried for up to three months still produced sporangia upon wetting (Davidson & Shaw 2003, *unpublished*). No work has been done on the effect of freezing on *P. ramorum* chlamydospores. Fichtner *et al.* (2005, *unpublished*) inoculated *Rhododendron* leaf discs with *P. ramorum* and left them for eight weeks in soil and at the soil/ litter interface. Leaf disks that initially had no fungal recovery on selective media were subsequently incubated in water for three weeks. The water incubation increased fungal recovery by 3 % in disks from soil and 10 % in disks from the drier soil/ litter interface on selective media. Fichtner *et al.* suggested that the recovery of *P. ramorum* after incubation in water coupled with high populations of chlamydospores in infected leaf tissue suggested the potential role of hydration in breaking chlamydospore dormancy (Fichtner *et al.* 2005, *unpublished*).

The leaf lesions exposed to 5 and 10 days of freezing and drying in this thesis were not subjected to a subsequent hydration treatment; it would be interesting to conduct that experiment. In a related experiment, however, *P. ramorum* infected leaf-lesions that had been frozen for 15 days were extracted from the leaf tissue, hydrated for 24 hours, and incubated on V8ARP; no chlamydospores germinated for six days.

It is important to understand the chlamydospore of *P. ramorum* as it occurs in *Rhododendron* leaves. This information, in conjunction with in-vitro studies of chlamydospore biology, is a necessary step in the understanding of the biological role of the chlamydospore in nature. The experiments presented here have documented the rate of growth of the fungus in detached *Rhododendron* leaves and tied that information with the findings of other workers. This is the first report of a chlamydospore incidence gradient within leaf lesions of *Rhododendron* infected with *P. ramorum*. Utilizing the findings of the chlamydospore incidence gradient, it is the first time that an approximate estimate of the total number of chlamydospores within a leaf lesion has been presented that is representative of the way it actually occurs within the leaf. These results have corroborated the finding of other workers that plant-grown chlamydospore walls are thicker in *Phytophthora* species than those grown in V8-based or other artificial media. This is the first report of a chlamydospore wall thickness as high as 8.0 µm (the thickest yet recorded) in *P. ramorum* or in the genus *Phytophthora*. This is also the first time that chlamydospore diameter has been shown to be related to wall thickness; this is true in leaf-, V8 agar-, and V8JB-grown chlamydospores. These results have verified the work of others which states that chlamydospores grown in *Rhododendron* leaves are capable of germination, but that the rates of germination are very low as they are from V8JB-grown chlamydospores. This is the first report of a negative impact of freezing on the recovery of *P. ramorum*, and it validates the negative impact of air-drying on recovery of the fungus on selective agar. The experiments presented here are an important first step in understanding the behavior of *P. ramorum* in *Rhododendron* leaves, but more detailed experiments must be conducted to better understand the biological role of the chlamydospore in *Rhododendron* and therefore in wildland ecosystems of southwestern Oregon and nursery environments.

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Chapter 5

Biology of Chlamydospores of *Phytophthora ramorum*: General Conclusion

Aaron Smith

Conclusion

The experiments presented here were conducted to better understand the biology of the chlamydospore of *P. ramorum*. These experiments covered the development and maturation of the chlamydospore in-vitro, its germination in-vitro, and how it behaves in-planta when grown in *Rhododendron* leaves. These experiments were designed to create a biological picture of the chlamydospore from its inception to germination or death. It was equally important to tie in-vitro results to in-planta results to better resolve the differences between chlamydospore behavior under laboratory conditions and behavior in nature. In pursuit of these goals, these experiments have brought to light a number of important results related to the biology of *P. ramorum* chlamydospores. The results are first briefly summarized and then followed by a more detailed discussion of their significance.

Large Diameter, Thick-Walled Chlamydospores

- *P. ramorum* produces abundant large diameter, thick-walled chlamydospores compared to many other *Phytophthora* species (Figures 3 & 4).

Chlamydospore Formation and Maturation

- Chlamydospore formation begins at two to three days on V8 agar; by four days some chlamydospores have fully formed (Figures 10 &12); within eight to ten days most chlamydospores are mature (Figures 10, 12, 14, & 16); they have fully expanded, formed a septum, and have formed a thickened inner wall.
- Chlamydospores are formed abundantly in lesions of detached *Rhododendron* leaves in a gradient pattern; fewer chlamydospores are present in more recently formed lesions than in lesions that have been infected longer (Table 13).
- Chlamydospores grown in *Rhododendron* leaves have thicker walls than chlamydospores grown in V8 agar or V8 juice broth (Table 14).

Chlamydospore Wall Thickening Over Time

- Some chlamydospore walls continue to thicken from ten to at least 120 days of age (4 months) (Figure 14, Table 3).

The Relationship Between Chlamydospore Diameter and Wall Thickness

- There is a strong relationship between chlamydospore diameter and wall thickness; chlamydospore wall thickness tends to increase with diameter in *P. ramorum* (Figures 18, 19, & 30).

Low and Variable Rates of Chlamydospore Germination

- *P. ramorum* chlamydospores grown in V8 juice broth (Tables 5, 6, 9, & 10) and *Rhododendron* leaves (Table 16) germinate at a low and variable rate.

Exogenous Stimulation of Chlamydospore Germination

- Chlamydospore germination in *P. ramorum* is controlled, in part, by the presence of exogenous nutrients (Tables 5, 6, & 16).

The Typical Germinated P. ramorum Chlamydospore

- Relatively smaller diameter, thinner-walled chlamydospores tend to germinate more often than larger diameter chlamydospores with thicker walls (Tables 7 & 8).

Effects of Freezing and Air-Drying on P. ramorum Recovery from Leaves

- Freezing or air-drying *P. ramorum*-infected *Rhododendron* leaves for 5, 10, and 15 days greatly reduces the recovery of the fungus on selective media (Tables 17 & 18).

Concluding Discussion

Large Diameter, Thick-Walled Chlamydospores

Chlamydospores are produced by at least 35 of the 75 described species of *Phytophthora.* Average chlamydospore diameters range from as low as 7.9 µm in *P. drechsleri* to as high as 67.5 µm in *P. quininea* (Figure 4). Average chlamydospore wall thickness across species in the genus is as low as 0.8 µm in several species to as high as 3.5 µm in *P. undulata* (Figure 3). *P. ramorum* has the thickest chlamydospore wall recorded for the genus at 8 µm for a Pacific rhododendron leaf-grown chlamydospore; interestingly the normally thin-walled (average 0.8 µm) *P. lateralis* has the second thickest chlamydospore wall recorded in the genus at 6-7 µm from agar and liquid media (Tucker & Milbrath 1942). Among *Phytophthora* species, *P. ramorum* produces a chlamydospore with the third largest average diameter (51.2 µm) and fourth thickest average wall thickness (2.4 µm) (Figures 3 & 4). *P. ramorum* has the second largest chlamydospore diameter recorded in the genus at 93 µm from V8JB

culture; second only to *P. cinnamomi* which had a chlamydospore as large as 135 µm recorded from naturally infested pineapple field soil (McCain *et al.* 1967). The average chlamydospore of *P. ramorum* is larger in diameter than the chlamydospores of 27 other *Phytophthora* species and has an average wall thickness that is thicker than 15 species (Figures 3 & 4).

It is important to understand how *P. ramorum* chlamydospore production and morphology relate to other chlamydospore-producing species. Such a comparison allows for the construction of a framework related to the known biology of the chlamydospores of other *Phytophthora* species. Some useful inferences can then be made regarding yet unknown, but important, aspects of the biology of *P. ramorum* chlamydospores. Although any inferences made to similarities in biology need to be empirically tested before true comparisons are made and not all aspects of chlamydospore biology are the same among *Phytophthora* species, there are several common threads that can be useful to workers. Species that produce abundant chlamydospores like *P. cinnamomi* (Shew 1980), *P. lateralis* (Hansen & Hamm 1996), and *P. palmivora* (Ramirez 1975) (Appendix A, Part 2) do so as a successful strategy to perpetuate themselves in nature through the use of the chlamydospore in effective strategies of epidemiology or survival. The chlamydospore in these species is an important component of their life history, as it likely is in *P. ramorum*, though much research needs to be done to fully understand its significance. The wall thickness of the chlamydospore is also important because it relates to what rates of germination can be expected from a thick- or thin-walled species (Kadooka & Ko 1973). Thick-walled chlamydospores of *P. palmivora* and *P. ramorum* both germinate at low rates (Kadooka & Ko 1973), whereas thin-walled chlamydospores have a higher rate of germination. Thinner walled species like *P. cinnamomi*, routinely germinate at high germination rates (Mircetich *et al.* 1968).

Chlamydospore Formation and Maturation

Chlamydospores of *P. ramorum* are formed abundantly and quickly in V8 agar, V8JB, and detached *Rhododendron* leaves. Chlamydospore formation begins as early as two to three days in V8 agar cultures (Figures 10 & 12) and detached *Rhododendron* leaves (Table 13). At four days of age V8 agar cultures contain many mature chlamydospores (i.e. expansion has stopped, a septum has formed, and inner wall thickening has begun) and the mean number of chlamydospores has risen to four chlamydospores/ mm^2 in leaves (Table 13). By eight to ten days of age most V8 agar and V8JB-grown chlamydospores have matured (Figures 10, 12, 14, & 16) and the number of chlamydospores in leaf lesions levels off to an average of about 23 chlamydospores/ mm^2 (Table 13). In 30 days of growth, the average V8 agar- and V8JB-grown chlamydospore is 50.8 µm and 54.0 µm in diameter respectively and has a wall thickness of 2.1µm , whereas leaf-grown chlamydospores are smaller in diameter (47.6 µm) with thicker walls (2.9 µm) (Tables 14 & 15).

A seamless description through time of chlamydospore maturation has not been presented in any *Phytophthora* species until now and it reveals an interesting pattern that has implications for further research. By ten days of age in V8 agar and V8JB cultures, most chlamydospores of *P. ramorum* are mature. This is important to know in the development of future research on chlamydospores because it allows the researcher to have a good idea of what diameter and, more importantly, what wall thickness the average chlamydospore encountered in the culture will likely have at a given time. This would allow for a more precise description of the chlamydospores that are being experimentally manipulated. A very important line of research utilizing this data would be to explore how the same pattern of chlamydospore maturation takes place in *Rhododendron* leaf-grown chlamydospores. This information, combined with a knowledge of the number of chlamydospores that are formed in leaf tissue (Table 13), the expected rates of chlamydospore survival (Table 17), expected rates of germination (Tables 5, 6, 9, 10, & 16), and the conditions under which each of these occur, would be very useful to understand the behavior of *P. ramorum* in wildland and nursery environments. This information could be directly applicable to controlling the disease in the future.

Another possibly important line of inquiry would be to use V8 agar-, V8JB-, and leaf-grown chlamydospores in a comparison within *P. ramorum* of the differing behavior of relatively thin-walled media-produced and thick-walled leaf-grown chlamydospores. Kadooka & Ko (1973) provided good evidence that germination rates are lower and heat tolerance is higher in thick-walled chlamydospores compared to thin-walled chlamydospores in *P. palmivora*. Likewise, chlamydospores of *P. ramorum* have also been shown to germinate at low rates and to be very heat tolerant (Harnik *et al.* 2004). With direct comparisons of chlamydospore survival and germination from media-produced and leaf-produced chlamydospores, workers would have a much better idea of the potential chlamydospore germination of *P. ramorum*.

Chlamydospore Wall Thickening Over Time

There is good evidence that in some chlamydospores of *P. ramorum*, the wall continues to thicken over a period of four months. Chlamydospores grown in V8JB at 20 C in the dark had a mean wall thickness of 2.3 µm in chlamydospores 20 to 120 days old, whereas the mean maximum wall thickness (i.e. only occurring in some chlamydospores) increased from ten to 120 days of age from 2.7 µm to 4.0 µm (Figure 14; Table 3). It is not clear if chlamydospore walls continue to thicken in leaf-grown chlamydospores, but there is some indication that the process differs in leaves. By 30 days of age the average leaf-grown chlamydospore had a mean wall thickness of 2.9 µm (maximum 8.0 µm) (Table 14) compared to the V8 agar thickness of 2.2 µm (maximum 4.8 µm) (Figure 10) and V8JB

thickness of 2.3 µm (maximum 4.3 µm) (Figure 14) indicating that chlamydospore walls thicken faster in leaf-grown chlamydospores.

A description of the continuation of wall thickening through time has not been presented in *P. ramorum* or in other *Phytophthora* species. Two important experiments to conduct would be to follow the continued wall thickening in some chlamydospores in V8 agar and V8JB-grown chlamydospores for more than the 120 days conducted in these experiments. Equally interesting would be to follow the wall thickening in some chlamydospores in leaf-grown chlamydospores. This would be important information to have to understand how the wall thickening process occurs in leaf-grown chlamydospores. Further studies could then explore survival and germination of these thicker-walled spores in *P. ramorum*.

The Relationship Between Chlamydospore Diameter and Wall Thickness

P. ramorum chlamydospore diameter tends to increase with chlamydospore wall thickness. In V8 agar-, V8JB-, and leaf-grown chlamydospores the relationship had r^2 values of 0.47 (n = 2100), 0.37 (n = 900), and 0.31 (n = 100) respectively (Figures 18 & 19; Figure 30). Though the r^2 values would indicate that the relationship is weaker in leaf-grown chlamydospores, this is likely due to the lower number (n) of chlamydospores sampled.

The relationship between chlamydospore diameter and wall thickness has not been recorded in other *Phytophthora* species. The relationship does, however, become apparent in a literature survey of thick-walled (> 1.5 µm) species; larger diameter species tend to have thicker walls and smaller diameter species tend to have thinner walls in the thicker-walled species (Figure 20). It is important to note that these are only single values for each species and do not reflect a description of the relationship as a process of chlamydospore maturation as described here. Among intermediate (1.0-1.5 µm) and thin-walled (< 1.0 µm) species, the relationship is not as clear. Among theses species, large diameter species such as *P. lateralis, P. insolita*, and *P. cinnamomi* have relatively thin chlamydospore walls (Figure 20). Conversely, the very small diameter *P. drechsleri*, has a wall thickness of 1.3 µm, relatively thick for such a small diameter spore. This relationship does not account for some very high maximum chlamydospore wall thickness values that have been recorded in some species (e.g. *P. lateralis*), instead it is based on average wall thickness values recorded for each species (Appendix A). It is not clear why thick-walled species exhibit this relationship more strongly than do thin-walled species in *Phytophthora*.

Low and Variable Rates of Chlamydospore Germination

P. ramorum chlamydospores germinate at a low and variable rate. In chlamydospores aged ten days in V8JB, germination on V8 agar after 24 hours of incubation ranged from a high of 12.9 % (Table 5) to a low of 0.2 % (Table 9) in different experiments. On cornmeal agar amended with antibiotics (CAR), the percent germination ranged from a high of 3.6 % to a low of 0.5 % (Table 5). On WA, percent germination ranged from 4.0 % to 0.3 % (Table 6). Among V8JB-grown chlamydospores germinated on V8 amended with antibiotics (V8ARP), percent germination was also low with an estimated 0.1 to 0.8 % germination (Table 10). Extracted *P. ramorum* chlamydospores grown for 28 days in *Rhododendron* leaves also germinated at low rates on V8ARP. Chlamydospore germination occurred over 12 days, but frequency of germination was unevenly distributed over that time. The number of germinated chlamydospores was five after two days of incubation at 20 C on agar, increased to seven after four and six days, and dropped to one chlamydospore every other day up to 12 days (Table 16). Each germinated leaf-grown chlamydospore formed a new fungal colony on the agar surface.

Chlamydospore germination in the *Phytophthora* species that have been studied is very often at much higher rates than the rates of chlamydospore germination in *P. ramorum* recorded here. The reason for the low and variable rates of chlamydospore germination in *P. ramorum* is likely related to the thick wall of the chlamydospore in *P. ramorum*. Kadooka & Ko (1973) provided the best evidence for the differing rates of germination found in thick-walled (5 %) compared to thin-walled (95 %) chlamydospores of *P. palmivora* on V8 agar. Kadooka & Ko's experiment highlights the importance of comparing the rates of germination of chlamydospores of differing wall thickness within a species and the reason that experiments with relatively "thin-" and thick-walled chlamydospores should be carried out in *P. ramorum*.

The biological significance of a *Phytophthora* species producing both thin- and thick-walled chlamydospores is not known. Clearly chlamydospore wall thickness has some relationship with the media in which the chlamydospore was formed; thinner in artificial media and thicker in plant tissue. Under both of these conditions some chlamydospores continue to develop thicker walls than the average chlamydospores in the population (Figures 10, 14, and 28). There is good evidence within *Phytophthora* that thicker-walled chlamydospores have increased resistance to microbial antagonism (Quitugua & Trujillo 1998), increased tolerance to heat (Harnik *et al.* 2004; Kadooka & Ko 1973), have lower rates of germination (Kadooka & Ko 1973), are more frequently produced in chlamydospores grown in plant tissue (Pogoda & Werres 2004), and are relatively scarce in numbers compared to the numbers of average chlamydospores produced (Figures 11, 15, & 28). There is also good evidence that thinner-walled chlamydospores are produced more frequently (Figures 11, 15, & 28) and germinate

more frequently after 24 hours of incubation on an agar surface than thick-walled chlamydospores (Tables 7 & 8).

It is possible that the thick- and thin-walled chlamydospores produced by the fungus represent simultaneous, but biologically different strategies to optimize their success in any given environment they are likely to encounter. Under this hypothesis, thin-walled chlamydospores (the majority of chlamydospores produced by a species) allow the fungus to more readily germinate when conditions conducive to germination and vegetative growth are encountered. The fewer thick-walled chlamydospores allow for the fungus to more likely survive longer periods over which time they are more likely to encounter a greater number of environmental extremes. It is also possible that thicker-walled spores are produced because of some undescribed physiological phenomenon. This hypothesis is strengthened by the finding that larger diameter chlamydospores tend to have thicker walls. This may simply be because there is more cytoplasm present within the chlamydospore and therefore more architectural material with which to construct a thicker wall. Countering this argument is the existence of relatively thin-walled, large diameter species such as *P. cinnamomi* (Figure 20). Neither of these hypotheses have been explored in any *Phytophthora* species, but it would be interesting to know the answer. In either case, a better understanding of the biological significance of thin- and thick-walled chlamydospores is a necessary avenue of future inquiry in *P. ramorum* and *Phytophthora* in general.

Exogenous Stimulation of Chlamydospore Germination

Chlamydospore germination is controlled, in part, by the presence of exogenous nutrients. In all experiments chlamydospore germination was significantly higher on nutrient-rich V8 agar than on CAR or WA. Average percent germination on V8 agar was 10.7 and 5.6 % (Tables 5 & 6), where as CAR was 2.4 % (Table 5), and WA was 1.7 and 1.5 % (Tables 5 & 6). Extracted leaf-grown chlamydospores also germinated better on nutrient-rich V8ARP compared to cornmeal agar amended with antibiotics (CARP) with a total of 22 germinated chlamydospores over 12 days on V8ARP and only one germinated chlamydospore by the fourth day and zero up to 12 days on CARP (Table 16).

Chlamydospore germination in *Phytophthora* is exogenous, that is it is controlled by external environmental conditions. In most species of *Phytophthora*, chlamydospore germination is enhanced by the presence of amino acids or exudates from plant roots (Khan & Reedleder 1996; Mircetich *et al.* 1968; Tsao & Bricker 1968). *P. palmivora* is unique in the genus, in that it does not require the presence of exogenous nutrients for high rates of germination, but it does require the presence of water (Chee 1973; Kadooka & Ko 1973). The presence of nutrients clearly enhances germination of *P. ramorum* chlamydospores, but it is not known which nutrients are necessary and in what concentration. Likewise, the effects of root exudates or plant tissue exudates on chlamydospore germination have not

been studied in *P. ramorum*. An important and fruitful line of future inquiry would be explore, more precisely, how the presence of different nutrients and plant exudates affects chlamydospore germination in *P. ramorum*. Such a study could have significant implications in the understanding of the biological role of the chlamydospore as it occurs in plant tissue and what its epidemiological potential is in natural and nursery environments.

The Typical Germinated P. ramorum Chlamydospore

In a population of ten-day-old V8JB-grown chlamydospores, smaller-diameter, thinner-walled chlamydospores are more likely to germinate. The average ten-day-old V8JB-grown chlamydospore has a diameter of 53.7 µm with a wall thickness of 1.8 µm (Tables 3 & 4). The average germinated V8JB-grown *P. ramorum* chlamydospore after 24 hours of incubation on V8 agar is 42.7 +/- 1.0 µm in diameter and has a wall thickness of 1.6 µm (Tables 7 & 8). This describes about 30 % of ten-day-old V8JB chlamydospores (Figures 15 & 17).

It is important to know first what are the morphological and physiological qualities of chlamydospores in a colony, It is then important to know which of those chlamydospores are likely to germinate, which are not in a given time interval, under what conditions germination is more likely to occur, and what the colony composition of each is. This information can be used to better define which chlamydospores are likely to germinate, over what time interval, and how they are able to survive differing conditions. If each of these parameters is better understood in the biology of *P. ramorum* chlamydospores, there is a much better chance at effectively mitigating the spread of the disease.

Effects of Freezing and Air-Drying on P. ramorum Recovery from Leaves

P. ramorum recovery from inoculated *Rhododendron* leaves is negatively affected by exposure to freezing or air-drying. There was no recovery of the fungus from lesions plated on CARP after five and ten days of freezing or air-drying 10 days of incubation at 20 C, whereas untreated inoculated leaves had 100 % recovery of the fungus (Table 17). In a related experiment, chlamydospores were extracted from lesions that had been frozen for 15 days, hydrated for 24 hours, and then plated on CARP; no germination was seen over six days (Table 18).

It is very important to understand what environmental conditions the chlamydospores of *P. ramorum* are capable of surviving. Chlamydospores are likely to encounter varying extremes of dryness, temperature, and microbial antagonism in natural environments both in plant tissue and free in soil. Given moist environments, chlamydospores of other *Phytophthora* species have been shown to survive for many years in sterile and non-sterile soil environments, though survival is better in sterile

conditions (Table 11). Exposure to cold appears to induce a winter dormancy in chlamydospores of other species and leads to a reduction of recovery of the fungus during colder winter months (Weste & Vithanage 1978). Air drying of chlamydospores seems to very quickly have a negative effect on the survival of the chlamydospore in other species (Erwin *et al.* 1983) and in *P. ramorum* (Davidson *et al.* 2002). More work is necessary to understand the specific conditions that chlamydospores of culture-grown and plant-grown chlamydospores of *P. ramorum* are capable of surviving. This knowledge is directly applicable to control measures that might be developed for the fungus.

The experiments presented here, taken together, represent an important first step in understanding the biological role of the chlamydospore in *P. ramorum* and highlight the necessity for further inquiry. It is clear that in other *Phytophthora* species the chlamydospore is an important survival mechanism capable of survival for many years in a field soil environment (Table 11). Chlamydospores of *P. ramorum* have been shown to survive for at least six months in a soil environment (Fichtner *et al.* 2005, *unpublished*; Linderman & Davis 2006; Shishkoff & Tooley 2004). Comparative information related to the capacity of the chlamydospore in other *Phytophthora* species to survive and wall thickness information would suggest that chlamydospores of *P. ramorum* are capable of surviving much longer periods than what has currently been reported. The fact that *P. ramorum* produces chlamydospores in great abundance and that the chlamydospore is the primary survival structure available to the fungus as it known to occur in wildland, interface, and nursery environments in North America and Europe, highlights the importance of having a clear understanding the biology of the chlamydospore in *P. ramorum.* Any meaningful disease mitigating measure is founded in a firm biological understanding of the organism. In the case of the chlamydospore in *Phytophthora* in general, and in *P. ramorum* in particular, there is much to learn. Further inquiries into the biology of the chlamydospore in *P. ramorum* are imperative if we are to have a mitigating effect on the future spread of the devastating disease it causes.

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APPENDICES

Appendix A Part 1: Published Average Chlamydospore Diameters and Wall Thicknesses for Different *Phytophthora* Species

Chlamydospore Producing *Phytophthora* Species	Average Chlamydospore Diameter (µm)	Chlamydospore Diameter Range (µm)	Average Wall Thickness (µm)	Wall Thickness Range (µm)	Reference (D) = Diameter (W) = Wall Thickness (Number corresponds to line referenced)
P. arecae	25.7 — 32.5	18.0-40.0 35.0-40.0 18.0-60.0	≤1.0 — —	— — —	D1: Tucker (1931), W1: Erwin & Ribeiro (1996) D2: Waterhouse (1963) D3: Gerrettson-Cornell (1989)
P. boehmeriae	41.4 29.5 40.0	26.0-51.0 17.0-42.0 30.0-41.0	2.0 — —	—	D1: Tucker (1931), W1: Erwin & Ribeiro (1996) D2: Frezzi (1950) D3: Gerrettson-Cornell (1989)
P. botryosa	18.7	14.0-30.0	—	—	D1: Chee (1969)
P. cactorum	31.6 33.2 33.0 34.5 39.7	23.0-43.0 19.0-53.5 53.0 max 14.0-55.0 28.0-56.0	1.3 — — — —	1.0-1.5 — — — —	D1: Tucker (1931), W1: Erwin & Ribeiro (1996) D2: Frezzi (1950) D3: Waterhouse (1963) D4: Gerrettson-Cornell (1989) D5: Darmano & Parke (1990)
P. capsici	28.5 37.0 23.8	28.0-29.0 22.0-52.0 20.0-27.5	2.6 1.3 2.3	2.4-2.7 1.0-1.5 2.0-2.5	D1 & W1: Tsao (1991) D2: Mchau & Coffey (1995), W2: Uchida & Aragaki (1985) D3 & W3: Islam *et al*. (2005)
P. cinnamomi	41.0 27.1 9.8 — 87.5	31.0-50.0 — 5.2-14.4 — 40.0-135.0	0.6 — 1.3 1.75 —	0.5-0.6 — 1.0-1.5 <1.0-2.6 —	D1: Rands (1922), W1: Hemmes & Wong (1975) D2: Erwin & Ribeiro (1996) D3 & W3: Cother & Griffin (1973) W4: Shew & Benson (1982) D5: McCain *et al*. (1967)
P. citricola	—	—	—	—	Erwin & Ribeiro (1996)
P. citrophthora	26.5 28.0	10.0-43.0 25.0-35.0	1.0 —	0.5-1.5 —	D1: Mchau & Coffey (1994), W1: Erwin & Ribeiro (1996) D2: Erwin & Ribeiro (1996)
P. colocasiae	27.0	17.0-38.0	2.5	2.0-3.0	D & W: Erwin & Ribeiro (1996)

Appendix A Part 1(Continued): Published Average Chlamydospore Diameters and Wall Thicknesses for Different *Phytophthora* Species

Chlamydospore Producing *Phytophthora* Species	Average Chlamydospore Diameter (µm)	Chlamydospore Diameter Range (µm)	Average Wall Thickness (µm)	Wall Thickness Range (µm)	Reference (D) = Diameter (W) = Wall Thickness (Number corresponds to line referenced)
P. cryptogea	–	–	–	–	Erwin & Ribeiro (1996)
P drechsleri	7.9	4.2-11.0	1.3	1.0-1.5	D & W: Cother & Griffin (1973, 1974)
P. ilicis	–	–	–	–	Erwin & Ribeiro (1996)
P. infestans	–	–	–	–	Erwin & Ribeiro (1996)
P. insolita	35.0	22.0-48.0	Thin	–	D & W: Ann & Ko (1980)
P. iranica	28.7	17.0-41.0	–	–	D: Erwin & Ribeiro (1996)
P. japonica ?	–	–	–	–	Erwin & Ribeiro (1996) (Table 4.2)
P. katsurae	15.6	12.0-19.2	–	–	D: Erwin & Ribeiro (1996)
P. lateralis	40.0 36.6	20.0-77.0 26.0-44.0	Thin –	6.0-7.0 –	D1 and W1: Tucker & Milbrath (1942) D2: Werres *et al*. (2001)
P. macrochlamydospora	55.0	24.0-84.0	3.3	2.5-4.0	D & W: Erwin & Ribeiro (1996)
P. meadii	23.0 30.5	16.0-30.0 19.9-38.1	– 1.6	– –	D1: Peries & Fernando (1966) D2 & W2: Dantanarayana *et al*. (1984)
P. medicaginis	–	–	–	–	El-Hamalawi & Erwin (1986, *unpublished*)
P. megakarya	30.0	20.0-40.0	–	–	D: Erwin & Ribeiro (1996)
P. megasperma	11.0	6.0-16.0	< 1.0	< 1.0-2.0	D & W: Basu (1980)
P. melonis	31.2	16.3-52.0	–	–	D: Katsura (1976)
P. mexicana	36.0	28.0-44.0	–	–	D: Erwin & Ribeiro (1996)
P. nicotianae (syn. P. parasitica)	28.0 33.0 40.0	13.0-60.0 – 20.0-60.0	–	–	D1: Hall (1993) D2: Hall (1993) D3: Dastur (1913)
P. palmivora	33.0 36.2 < 25.0 40.0 32.5	32.0-42.0 30.0-45.0 – 24.9-48.1 30.0-35.0	> 1.5 – – 3.6 0.8	0.5->1.5 – – 3.3-3.9 0.3-1.2	D1: Holiday (1980), W1: Kadooka & Ko (1973) D2: Erwin & Ribeiro (1996) D3: Erwin & Ribeiro (1996) D4 & W4: Dantanarayana *et al*. (1984) D5 & W5: Hemmes & Lerma (1985)

Appendix A Part 1(Continued): Published Average Chlamydospore Diameters and Wall Thicknesses for Different *Phytophthora* Species

Chlamydospore Producing *Phytophthora* Species	Average Chlamydospore Diameter (µm)	Chlamydospore Diameter Range (µm)	Average Wall Thickness (µm)	Wall Thickness Range (µm)	Reference (D) = Diameter (W) = Wall Thickness (Number corresponds to line referenced)
P. polygoni	16.0	12.3-24.6	0.8	0.5-1.0	D & W: Zheng & Ho (2000)
P. porri	30.0	20.8-35.3	–	–	D: Erwin & Ribeiro (1996)
P. quininea	67.5	45.0-90.0	–	–	D: Erwin & Ribeiro (1996)
P. ramorum	47.6	19.0-80.0	2.9	0.8-8.0	D1 & W1: Smith MS Thesis (2007) (Leaf-grown)
	50.8	15.5-80.5	2.1	0.5-4.8	D2 & W2: Smith MS Thesis (2007) (V8 Agar-grown)
	54.0	21.0-93.0	2.1	0.6-4.3	D3 & W3: Smith MS Thesis (2007) (V8JB-grown)
	52.5	20.0-91.0	–	–	D4: Werres *et al*. (2001)
P. sojae	30.0	–	–	–	D: Hildebrand (1959), Erwin & Ribeiro (1996)
P. syringae	25.0	–	–	–	D: Stamps *et al*. (1990)
P. tentaculata	26.6	10.0-45.0	Thin	–	D & W: Erwin & Ribeiro (1996)
P. undulata	36.0	21.0-61.0	3.5	2.0-5.0	D & W: Peterson (1910); Van der Plaats-Niterink (1981)
P. vignae	17.0	12.0-21.0	–	–	D: Erwin & Ribeiro (1996)

Appendix A Part 2: Published Chlamydospore Frequency, Abundance, Production Variation by Isolate, and Time Until Chlamydospores are Produced Descriptions for Different *Phytophthora* Species

Chlamydospore Producing *Phytophthora* Species	Frequency of Chlamydospore Production	Chlamydospore Abundance	Chlamydospore Production Varies by Isolate	Time Until Chlamydospores Produced	Reference (F) Frequency of Chlamydospore Production (A) Chlamydospore Abundance (V) Isolate Variation (T) Time Until Chlamydospores Produced
P. arecae	Infrequent	–	Yes	–	F & V: Erwin & Ribeiro (1996)
P. boehmeriae	Infrequent	–	–	–	F: Erwin & Ribeiro (1996)
P. botryosa	Infrequent	Sparse	–	–	F: Erwin & Ribeiro (1996); A: Chee (1969)
P. cactorum	Frequent or Infrequent	Abundant in some isolates and erratic in others	Yes	–	F, A, & V: Erwin & Ribeiro (1996)
P. capsici	Frequent or Infrequent	Abundant in some isolates and rare in others	Yes	4-8 weeks	F, A, & T: Uchida & Aragaki (1985) A & V: Tucker (1931); Alizadeh & Tsao (1985); Tsao (1991)
P. cinnamomi	Frequent	Abundant in all isolates	No	24-72 hours	F, A, & V: Erwin & Ribeiro (1996) T: Cahill *et al.* (1989)
P. citricola	Infrequent	–	–	–	F: Erwin & Ribeiro (1996)
P. citrophthora	Infrequent	–	Yes	3 weeks	F, V, & T: Mchau & Coffey (1994)
P. colocasiae	Frequent or Infrequent	Abundant in some isolates and rare in others	Yes	–	F, A, & V: Erwin & Ribeiro (1996)
P. cryptogea	Infrequent	–	–	–	F: Erwin & Ribeiro (1996)
P drechsleri	Infrequent	–	Yes	–	F & V: Frezzi (1950); Cother & Griffin (1973, 1974)

Appendix A Part 2 (Continued): Published Chlamydospore Frequency, Abundance, Production Variation by Isolate, and Time Until Chlamydospores are Produced Descriptions for Different *Phytophthora* Species

Chlamydospore Producing *Phytophthora* Species	Frequency of Chlamydospore Production	Chlamydospore Abundance	Chlamydospore Production Varies by Isolate	Time Until Chlamydospores Produced	Reference (F) Frequency of Chlamydospore Production (A) Chlamydospore Abundance (V) Isolate Variation (T) Time Until Chlamydospores Produced
P. ilicis	Infrequent	–	–	–	F: Erwin & Ribeiro (1996)
P. infestans	Only once	–	–	4-9 months	F & T: Patrikeyeva (1979)
P. insolita	–	–	–	–	Ann & Ko (1980)
P. iranica	Infrequent	–	–	–	F: Erwin & Ribeiro (1996)
P. japonica ?	–	–	–	–	Erwin & Ribeiro (1996) (Table 4.2)
P. katsurae	–	–	–	–	Erwin & Ribeiro (1996)
P. lateralis	Frequent	Abundance varied among isolates	–	–	F: Erwin & Ribeiro (1996) A:Trione (1974)
P. macrochlamydospora	Frequent	–	–	–	F: Erwin & Ribeiro (1996)
P. meadii	Infrequent	–	–	> 18 days	F & T: Peries & Fernando (1966)
P. medicaginis	Infrequent	–	–	–	F: El-Hamalawi & Erwin (1986, *unpublished*)
P. megakarya	–	–	–	–	Erwin & Ribeiro (1996)
P. megasperma	–	–	–		Basu (1980)
P. melonis	–	Not always present	–	–	A: Katsura (1976); Ho *et al*. (1984)
P. mexicana	Infrequent	–	–	–	Erwin & Ribeiro (1996)
P. nicotianae (syn. P. parasitica)	Frequent or Infrequent	Abundant in most isolates	Yes	–	F, A, & V: Erwin & Ribeiro (1996); Hall (1993)
P. palmivora	Frequent or Infrequent	Abundant in most isolates and not formed in others	Yes	5 days	F, A & V: Mchau & Coffey (1994) T: Hemmes & Lerma (1985)

Appendix A Part 2 (Continued): Published Chlamydospore Frequency, Abundance, Production Variation by Isolate, and Time Until Chlamydospores are Produced Descriptions for Different *Phytophthora* Species

Chlamydospore Producing *Phytophthora* Species	Frequency of Chlamydospore Production	Chlamydospore Abundance	Chlamydospore Production Varies by Isolate	Time Until Chlamydospores Produced	Reference (F) Frequency of Chlamydospore Production (A) Chlamydospore Abundance (V) Isolate Variation (T) Time Until Chlamydospores Produced
P. polygoni	–	Abundant	–	12-15 days	A & T: Zheng & Ho (2000)
P. porri	–	–	–	8 weeks	T: Erwin & Ribeiro 1996
P. quininea	Frequent	Abundant in all isolates	–	5.5 days	F, A, & T: Erwin & Ribeiro (1996)
P. ramorum	Frequent	Abundant in all isolates	No	4 days	F: Werres *et al*. (2001) A, V, & T: Smith MS Thesis (2007)
P. sojae	Infrequent	–	–	–	F: Kaufmann & Gerdemann (1958); Hildebrand (1959)
P. syringae	Infrequent	–	–	–	F: Waterhouse & Waterston (1964); Stamps *et al.* (1990)
P. tentaculata	–	–	–	–	Erwin & Ribeiro (1996)
P. undulata	–	–	–	–	Peterson (1910); van der Plaats-Niterink (1981)
P. vignae	–	Sparse	–	–	A: Erwin & Ribeiro (1996)

Appendix A Methods: The range of values were constructed using the low and high diameter and wall thickness recorded value. Average values were either calculated from published ranges or written as published averages. Species that had the qualifiers "thin" or "< 1.0" were assigned a value of 0.8 µm (*P. insolita, P. lateralis, P. megasperma*, and *P. tentaculata*). Species that had a qualifier of "≤ 1.0" were assigned a value of 1.0 µm (*P. arecae*). The average value for a given species is an average of calculated or published averages for the species.

Appendix B: Agar and Liquid Media

CAR (Corn Meal Agar plus Ampicillin and Rifampicin)

- 17.0 g CMA
- 1.0 L DIH_2O
- Autoclave 30 minutes
- Let cool until ready to pour
- 200.0 mg Ampicillin
- 10.0 mg Rifampicin dissolved in 3.0 ml methanol at 20 C

CARP (Corn Meal Agar plus antibiotics)

- 17.0 g CMA
- 1.0 L DIH_2O
- Autoclave 30 minutes
- Let cool until ready to pour
- 20.0 mg Delvocid
- 200.0 mg Ampicillin
- 10.0 mg Rifampicin dissolved in 3.0 ml methanol at 20 C

CMA (Corn Meal Agar)

- 17.0 g CMA (Corn Meal Agar; BD brand, BBL™)
- 1.0 L DIH_2O

V8 Stock

- 1.43 g $CaCO_3$ (Calcium carbonate, Sigma®) : 100.0 ml V8 Juice (Campbell Soup Company, Camden, NJ, USA)
- Centrifuge (Hermile Labnet Centrifuge, model Z 383) 15 minutes at 2000 RPM
- Collect supernatant (i.e. V8 stock)
- Store frozen

Clarified V8 Stock

- V8 stock was filtered three times through single layer of Miracloth (Calbiochem) placed on a Buchner ceramic funnel (CoorsTek® #60242).
- The Miracloth filter was rinsed clean under running DIH_2O between each filtering.

V8 Agar

- 150.0 ml Clarified V8 Stock
- 850.0 ml DIH_2O
- 30.0 mg β-sitosterol (Acros organics, β-sitosterol with ca. 10% capesterol and ca. 75% β-sitosterol) dissolved in 5.0 ml boiling ethanol
- 17.0 g Bacto Agar (Bacto™ Agar, BD: Brandname)
- Autoclave 30 minutes

V8JB (V8 Juice Broth)

- 100.0 ml Clarified V8 Stock
- 900.0 ml DIH_2O
- 30.0 mg β-sitosterol dissolved in 5.0 ml boiling ethanol
- Autoclave 30 minutes
- Allow to cool to 20 C

V8ARP (V8 Agar plus antibiotics)

- 150.0 ml Clarified V8 Stock
- 850.0 ml DIH_2O
- 30.0 mg β-sitosterol dissolved in 5.0 ml ethanol
- 17.0 g Bacto Agar
- Autoclave 30 minutes
- Let cool until ready to pour
- 20.0 mg Delvocid (DSM Food Specialties, 50 % natamycin and sodium chloride)
- 200.0 mg Ampicillin
- 10.0 mg Rifampicin dissolved in 3.0 ml 20 C methanol

WA (Water Agar)

- 17.0 g Bacto Agar
- 1.0 L DIH_2O
- Autoclave 30 minutes