REPEATED EMERGENCE, MOTILITY, AND AUTONOMOUS DISPERSAL BY SPORANGIAL AND CYST DERIVED ZOOSPORES OF *PHYTOPHTHORA* SPP.

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CHAPTER I

INTRODUCTION

Phytophthora spp. are zoosporic plant pathogens responsible for a number of plant diseases of commercially important crops worldwide (Erwin *et al*., 1983; Erwin and Ribeiro, 1996; Agrios, 1997). The occurrence of phytopathogenic *Phytophthora* spp. in irrigation water has been known since the pioneering work of Bewley and Buddin (1921). *Phytophthora* zoospores or structures produced by them (cysts, hyphal fragments, appressorium-like structures, microsporangia) do occur and survive in irrigation water (Thomson, 1972 and Duniway, 1979). In addition, these oomycetous plant pathogens have a complex asexual life cycle with distinct multiple infectious propagules which include mycelium, sporangia, zoospores, and zoospore cysts (de Souza *et al*., 2003). All these asexual life cycle stages are influenced differently by environmental factors such as temperature, water relations, physical and chemical conditions, and with interacting combinations of these factors (Fawcett, 1936; Zentmeyer, 1981; Duniway, 1983). The geographic distribution of *Phytophthora* and the diseases they cause depend on the constraints of these environmental factors on any or all of the life cycle stages (Duniway, 1983). Among the environmental factors, temperature has been reported to have a large influence on growth, reproduction, and pathogenesis of *Phytophthora* spp.

(Sujkowski, 1987; Sing and Chauhan, 1988; Matheron and Matejka, 1992). Effects of temperature on soilborne *Phytophthora* spp. have been reported (Zentmeyer and Erwin, 1970; Zentmeyer, 1981; Sujkowski, 1987; Matheron and Matejka, 1992). However, these studies take into account only one species or isolate of *Phytophthora* and consider only one particular stage in the life cycle (Gooding and Lucas, 1959; Zentmeyer and Erwin, 1970; Zentmeyer, 1981; Sujkowski, 1987; Matheron and Matejka, 1992; Sato, 1994; Roy, 1999; Timmer *et al.*, 2000). This study examined the effect of temperature on the asexual life cycle stages, which included the mycelial growth, sporangia production, and zoospore cyst germination of several water-borne *Phytophthora* spp.

The predominant propagules of *Phytophthora* spp. in wet and irrigated environments are the motile biflagellate zoospores (Thomson, 1972; von Broembsen and Charlton, 2001). These are produced in asexual sporangia borne on hyphal tips of the mycelium. The released zoospores swim in films of water in search of a host and with the aid of a combination of tactic signals, and locate suitable infection sites (Gow *et al.,* 1999; van West *et al*., 2002, 2003). Thus, zoospore competency as plant infective units requires that they undergo a characteristic multi-stage pre-infection sequence involving taxis, docking, encystment, cyst adhesion, and cyst germination (Deacon and Donaldson, 1993; Deacon, 1996). The zoospore cysts, depending on environmental cues have different options. They have the ability to remain encysted, germinate by germ tubes or by releasing additional zoospores (repeated emergence) (von Broembsen and Deacon, 1997; Xu and Morris, 1998; von Broembsen and

Charlton, 2000). The zoospores that emerge from the cysts during re-emergence (cyst derived zoospores) are structurally similar to zoospores released from the sporangia (sporangial derived zoospores). The released cyst derived zoospore wiggles its way out of the cyst, leaving the cyst cell wall (ghost) behind with or without a prominent exit tube (Fig. 3.1). The ecological significance, if any, of repeated emergence in natural conditions is not known. This unique phenomenon is thought to be a survival strategy in the absence of immediately available hosts by allowing cysts to delay germination until more favorable circumstances and then releasing further zoospores to achieve infection (Cerenius and Söderhäll, 1985; von Broembsen and Deacon, 1997). This study was undertaken to document the prevalence and occurrence of repeated emergence among water-borne *Phytophthora* spp. Understanding zoospore repeated emergence and factors that influence it, especially in water-borne *Phytophthora* spp., is an essential step in understanding the epidemiology of these pathogens. *Phytophthora* zoospores depend entirely on endogenous energy reserves (Cerenius and Söderäll, 1984; Pennington *et al*., 1989), which cannot be renewed. They use their endogenous energy reserves for motility, autonomous dispersal, and physiological activities such as maintaining appropriate balance with their surrounding media. Theoretically, this implies that cyst derived zoospores are likely to be depleted of energy having spent some of their macromolecules in cyst wall development and formation of new flagella. Cyst derived zoospores are expected to be less biologically fit as dispersive, multiplicative and primary infective units of *Phytophthora* spp. compared to

sporangial derived zoospores. However, the biological attributes of these different generations of zoospores including the ability to disperse autonomously, infect roots, and retain motility remain unknown.

CHAPTER II

LITERATURE REVIEW

BACKGROUND INFORMATION

The zoosporic organisms, which were traditionally classified as fungi by mycologists, are a taxonomically diverse group whose only justification for being grouped together is the production of flagellated zoospores (Barr, 1983). The current classification system (Alexopoulos *et al.,* 1996) places these organisms into six different Phyla; Myxomycota, Labyrinthulomycota, Chytridiomycota, Hyphochytri[di]omycota, Plasmodiophoromycota, and Oomycota (Barr, 1983; Alexopoulos *et al*., 1996). Delimitation of the zoosporic fungi into these taxa is made on the bases of zoospore flagellation (Barr, 1983; Fuller and Jaworski, 1987) and modes of infection. Thus, Chytridiomycota have a single posterior whiplash (smooth) flagellum and have close affinity to true fungi (Fuller and Jaworski, 1987). Hyphochytriomycota have a single anterior, tinsel flagellum, whereas, Plasmodiophoromycota have two anterior whiplash flagella of unequal length and share many features with protozoa. Oomycota have anterior tinsel and posterior whiplash flagella, which are of unequal length. Zoospores, aptly described by Fuller (1977) as the “hallmark of the aquatic fungi”, are adapted for survival in aquatic environment (McIntosh, 1972), thereby in turn making zoosporic fungi better adapted to the aquatic environment (Duniway, 1979). This has enabled zoosporic fungi to have a widespread occurrence in

aquatic habitats (Klotz *et al*., 1959; McIntosh, 1966; Thomson, 1972; Thomson and Allen, 1974; Kliejunas and Ko, 1976; Shokes and McCarter, 1979; Pittis and Colhoun, 1984; von Broembsen, 1984; Mircetich *et al*., 1985; Pottorf and Panter, 1997). However, the term “aquatic fungi” is an inappropriate reference to zoosporic fungi (Barr, 1983), since many zoosporic fungi are not primarily aquatic and occur in soil and many are foliar plant pathogens. In addition, there are many non-zoosporic fungi found in the aquatic habitats (Thomson, 1972). Much of the available literature on zoosporic fungi is based on saprophytic species because these are easier to study, while many zoosporic plant pathogens are obligate parasites, which do not lend themselves to ecological and biological investigations. Of the zoosporic plant pathogens, the oomycete species from genera *Pythium* and *Phytophthora* are of particular concern to agricultural producers because they are well adapted to aquatic survival, occur and disperse in irrigation waters, and are responsible for many diseases. Therefore, the formulation of suitable management approaches of these phytopathogens will depend on a sound understanding of their ecology and biology in water.

Taxonomic position of Oomycetes. The traditional five-kingdom classification system of Whittacker (1969) placed the oomycetes in the Kingdom Fungi due to their filamentous growth habit. However, more recent knowledge gleaned from molecular and biochemical analyses indicate unambiguously that the oomycetes are evolutionarily distinct and distantly related to true fungi (Chytridiomycota, Zygomycota, Ascomycota and Basidiomycota), although they produce vegetative

and reproductive structures that resemble those of the true fungi (Barr, 1983, 1992; Alexopoulos *et al*., 1996; van West *et al*., 2003). Oomycetes are 'fungus-like' mycelial organisms currently classified in the eukaryotic kingdom Straminipila (Alexopoulos *et al*., 1996; Dick, 2001) and are more closely related to the heterokont algae (Chrysophyceae, Phaeophyceae, and Xanthophyceae (Barr, 1992; Govers, 2001) than true fungi. Dick (2001) favored the kingdom name 'Straminipila' over Cavalier-Smith's (1986) earlier published kingdom name 'Chromista' because the anteriorly directed straminipilous flagellum is the critical diagnostic feature of this kingdom and not the presence of pigmented organelles, chromophyte endosymbionts, which formed the basis of Cavalier-Smith's Kingdom name Chromista (Cavalier-Smith, 1986). The remarkable distinguishing features of oomycetes include production of heterokont (one tinsel and one whiplash) dispersive zoospores, sexual oospores, possession of β-1, 3-glucan polymers, and cellulose as predominant cell wall constituents (Erwin *et al*., 1983). They exhibit vegetative diploidy and have mycolaminarin as their storage carbohydrates (Erwin *et al*., 1983; Erwin and Ribeiro, 1996; Margulis and Schwartz, 2000). Oomycetes (Kingdom: Straminipila) comprise three recognizable subclasses: Saprolegniomycetidae, Rhipidiomycetidae, and Peronosporomycetidae. Saprolegniomycetidae contain many oomycetes pathogenic to fish such as *Saprolegnia* as well as the important plant pathogenic species in the genus *Aphanomyces (*Bruno and Wood, 1999). However, most of plant pathogenic oomycetes belong to two orders: Peronosporales and Pythiales of the subclass Peronosporomycetidae (van West *et al*., 2003). The latter order

includes obligate biotrophic pathogens such as *Plasmopara*, *Albugo*, *Bremia*, and *Peronospora* spp. that cause downy mildew and white rust on several economically important crops (Agrios, 1997). The order Pythiales contains the family Pythiaceae that comprises economically important genera of *Phytophthora* and *Pythium* (Alexopoulos *et al*., 1996; Agrios, 1997). All these genera are reputed to be responsible for annual losses of many valuable food and cash crops, ornamental plants, and forest species worldwide (Erwin and Ribeiro, 1996; van West *et al*., 2003; Appiah *et al*., 2005).

The genus *Phytophthora*. The genus name *Phytophthora*, derived from Greek words *Phyton* and *phthora*, literally means 'plant destroyer' and was coined by Anton de Bary in 1876 to describe the type species *Phytophthora infestans* (Mont.) de Bary. The term 'plant destroyer' is a befitting description of the causal agent of potato late blight causal organism, which devastated potato production in the mid 19th century in Ireland (1845 – 1846), and still remains a worldwide production constraint nearly 160 years later. The genus *Phytophthora* is cosmopolitan and with over 67 species described (Erwin and Ribeiro, 1996), is arguably one of the most devastating group of plant pathogens (Erwin *et al*., 1983; Erwin and Ribeiro, 1996; Agrios, 1997). They cause enormous economic losses on several important food, fiber, and ornamental crops worldwide (Erwin and Ribeiro, 1996). According to Kamoun (2003), "virtually every dicotyledonous plant is affected by one or more species of *Phytophthora*, and several

monocotyledonous species are infected as well". They cause root and crown rots, trunk cankers, foliar, twig, and fruit blights (Agrios, 1997).

In the traditional taxonomy of Waterhouse (1963) and Stamps *et al.* (1990), Phytophthoras were divided into six morphological groups on the basis of sporangium structure (nonpapillate, semipapillate, or papillate), form of antheridium (amphigynous or paragynous), and whether the taxon is inbreeding (homothallic) or outbreeding with A_1 and A_2 mating types (heterothallic). Amphigyny is exclusively a feature of heterothallic species whereas homothallic species either exhibit amphigyny or paragyny or, in some cases, produce antheridia of both types (Cooke *et al*., 2000). These morphological groups are in no way a reflection of natural relationships among *Phytophthora* spp. but they nevertheless still form indispensable identification keys to phytophthorologists.

***Phytophthora* disease cycle.** *Phytophthora* spp. are responsible for a number of commercially important plant diseases (Erwin *et al*., 1983; Erwin and Ribeiro, 1996; Agrios, 1997). The infective units of these oomycetous plant pathogens include zoospores, oospores, chlamydospores, sporangia, and hyphal fragments (Thomson, 1972). However, for majority of *Phytophthora* spp. zoospores constitute the dominant infection units (Thomson, 1972; von Broembsen and Charlton, 2001) responsible for explosive disease epidemics. After release from sporangia, motile biflagellate zoospores are either moved passively or swim actively in water (Duniway, 1976) to potential infection sites. They are chemotactically attracted to elongation zones of roots of potential host plants

where they settle and encyst (Zentmeyer, 1961; Ho and Zentmeyer, 1977; Hardham, 2001). Zoospore cysts usually germinate within 20 – 30 min after encystment (Hardham, 2001) and germ tube grows and penetrates plant epidermal cells intercellularly along the anticlinal cell walls. As colonization advances, the pathogen produces haustoria that ramify in the cortical cells, acquire nutrients from plant, produce chlamydospores in cortical cells, and multinucleate sporangia on the root surface within 2 – 3 days (Ho and Zentmeyer, 1977; Hardham, 2001). The cytoplasmic contents of sporangia undergo cytokinesis to form uninucleate motile zoospores that are released through an apical pore in the sporangium. After discharge, the new generations of motile zoospores seek out and accumulate at new infection sites (Deacon and Donaldson, 1993) and the infection cycle is repeated.

Occurrence of *Phytophthora* spp. in irrigation water. Occurrence of phytopathogenic *Phytophthora* spp. in irrigation water was first reported by by Bewley and Buddin in 1921. Since then, there have been several reports of successful recovery of *Phytophthora* spp. from water bodies including nursery irrigation water (Klotz *et al*., 1959; McIntosh, 1966; Thomson and Allen, 1974; Kleijunas and Ko, 1976; Shokes and McCarter, 1979; Ali-Shtaye *et al*., 1991; Pittis and Colhoun, 1984; von Broembsen, 1984, 1990; MacDonald *et al*., 1994; von Broembsen and Wilson, 1998; Yamak *et al*., 2002; Bush *et al*., 2003). *Phytophthora* spp. have been noted to occur in water bodies such as puddles, runoff water in streams (Kleijunas and Ko, 1976; Shokes and McCarter, 1979

and Ali-Shtaye *et al*., 1991), in lakes and rivers (von Broembsen, 1984, 1990) and in irrigation water (Klotz *et al*., 1959; Thomson and Allen, 1974; von Broembsen and Wilson, 1998; Yamak *et al*., 2002). Thomson and Allen (1974) isolated various plant pathogens from irrigation tail water in Arizona. Their isolations included *Phytophthora parasitica* Breda de Haan (Dastur) and *P. citrophthora* (R.E. Sm. & E.H. Sm.) Leonian. Klotz *et al.* (1959) recovered *Phytophthora* spp. from canals and reservoirs that served as sources of irrigation water to citrus growers in California. von Broembsen (1990) established the occurrence of *Phytophthora cinnamomi* Rands in river waters used for irrigation in Southwestern Cape Province of South Africa. In separate studies, MacDonald *et al.* (1994) and von Broembsen and Wilson (1998) recovered several *Phytophthora* spp. namely; *Phytophthora cryptogea* Pethybr. & Lafferty, *P. citrophthora* (R.E. Sm. & E.H. Sm.) Leonian, *P. citricola* Sawada, *P. cinnamomi* Rands, and *P. parasitica* Breda de Haan (Dastur) from ornamental nursery irrigation run-off. The pathogenicity of the pythiaceous fungi isolated from water to commercially grown plants irrigated with these waters has been confirmed (Pittis and Colhoun, 1984; Ali-Shtaye *et al*., 1991; Yamak *et al.,* 2002) and, in most cases, a strong correlation has been reported between the use of infested water and diseases in the nursery industry (Macdonald *et al*., 1993). Between 1961 and 1963, McIntosh (1966) found *Phytophthora cactorum* (Lebert & Cohn) J. Schröt*,* the cause of collar rot of fruit trees in the water of many irrigation systems in the Okanagan and Similkaneen valleys of British Columbia. Yamak *et al.* (2002) also reported the occurrence of pathogenic *Phytophthora* spp.

including *P. cactorum* in the irrigation water in the Wenatchee river valley of Washington. Additionally, the following *Phytophthora* spp. have also been recovered in water assays by different workers: *P. palmivora* (E.J. Butler) E.J. Butler (Ali-Shtaye *et al*., 1991), *P. syringae* (Kleb.) Kleb (Klotz *et al.,* 1959), *P. megasperma* Drechs. (MacDonald *et al.,* 1994), *P. cambivora* (Petri) Buisman (McIntosh, 1966), *P. gonapodyides* (Petersen) Buisman (Pittis and Colhoun, 1984), *P. drechsleri* Tucker, and *P. capsici* Leonian (Bush *et al*., 2003). *Phytophthora* spp. usually survive in these aquatic environments as oospores, chlamydospores, microsporangia, hyphal fragments or as zoospores (Thomson, 1972). The first three mentioned propagule types usually germinate into sporangia, which ultimately produce zoospores (Pittis and Colhoun, 1984) if conditions are favorable. According to Thomson and Allen (1976), *Phytophthora* sporangia are stimulated to readily release zoospores in water. Therefore, zoospores constitute the dominant *Phytophthora* propagules that occur in irrigation water (Thomson, 1972; von Broembsen and Charlton, 2001) and are thought to be responsible for disease outbreaks when zoospore contaminated or recycled irrigation water is used on susceptible nursery crops.

***Phytophthora* zoospores as inoculum in irrigation water**. Zoospores of *Phytophthora* spp. survive and are particularly adapted to the aquatic environment (McIntosh, 1972; Duniway, 1979), where they serve the dual function of initiation of new generations and of dissemination of the pathogen (Hickman and Ho, 1966). These functions are critical in the life cycle of

Phytophthora as the zoospore must be able to gain access to the substrate, survive, and be easily dispersed to the susceptible hosts. The spread of these zoosporic organisms by means of zoospores depends on water availability, both for free swimming, and in the final phases for the formation and discharge of zoospores (Lange and Olson, 1983). Water can also be critical for encystment and host penetration by the encysted zoospore. Thus, free water is an important factor in the epidemiology of the zoosporic plant pathogens as it aids in flagellated zoospore movement. The distance traveled by the individual zoospores is usually relatively short (Lange and Olson, 1983), and is very much a function of the duration of active swimming period, which in turn is governed by the following factors: (i) cessation of motility due to the presence of a suitable substrate, which induces encystment, and initiation of infection, (ii) the exhaustion of energy supply/potential carried by the zoospore, and (iii) zoospore encounter with extreme conditions, which in some way leads to its lysis. However, where natural water flow or passive water flow occurs through the soil due to irrigation or rainfall, zoospores may spread over long distances or over neighboring fields (Erwin *et al*., 1983; Ristaino *et al*., 1988). The dispersed zoospores encyst, marking the final endpoint of the motile period for the zoosporic phytopathogens. If a suitable infection site on a host is located, then this encystment may at the same time initiate host penetration.

Sources of *Phytophthora* zoospores that contaminate irrigation water. The oospores or chlamydospores that occur in the sediment or plant debris at the

bottom of water reservoirs or in surface floating plant debris can serve as sources of zoospores in the waters (Pittis and Colhoun, 1984). These structures usually germinate to produce sporangia, which ultimately release zoospores under favorable environmental conditions. Owing to negative geotaxis (Cameron and Carlile, 1977; Charlton, 2001), the released zoospores swim upwards (against gravity) in water to occupy positions where passive dispersal is more likely. Cameron and Carlile (1977) while working with *Phytophthora cactorum*, *P. nicotianae,* and *P. palmivora* demonstrated that zoospores are negatively geotropic and accumulate at the uppermost surface of the water. Active swimming and not flotation cause the negative geotropism, as *Phytophthora* zoospores have a higher density than the water. In addition, the asymmetrical nature of the *Phytophthora* zoospores in terms of flagella position and their shapes is a contributing factor. Ho *et al.* (1968a) reported that *Phytophthora* zoospores are broader at the posterior than the anterior. This morphological feature makes the zoospore more inclined to point slightly upward as it swims, and show a net tendency to swim to the surface.

The other important source of zoospores in irrigation waters is the irrigation water itself. Water flowing over infested soils or crops usually moves zoospores or encysted zoospores and deposits them downstream. However, of the many important zoospore inoculum sources mentioned in the literature, recirculated irrigation water is the most important one. This is because the running irrigation water has the potential of moving the inoculum (zoospores, cysts, oospores or chlamydospores) from a small number of infected plants and

then releases large numbers of the same type of inoculum when recirculated back to the susceptible plants (MacDonald *et al*., 1994).

Survival of *Phytophthora* zoospores in irrigation water. *Phytophthora* zoospores or structures produced by them (cysts, hyphal fragments, appressorium-like structures, and microsporangia) are particularly adapted for survival in irrigation water (Thomson, 1972; Duniway, 1979). Thomson and Allen (1976) observed motile zoospores in water preparations for up to 20 hours. They also reported that zoospores of *Phytophthora parasitica*, or structures produced by them, survived for 40 – 60 days in irrigation water. However, they did not determine whether the source of the observed zoospores was from sporangia or zoospore cysts. Zoospore cysts exposed to conditions stimulatory to germination but unconducive for vegetative growth usually form short hyphal fragments as survival structures (Thomson, 1972). Production of microsporangia is a further survival mechanism (Thomson, 1972; Thomson and Allen, 1976). Thomson (1972) reported that microsporangia can survive for over 60 days in irrigation water. However, they never quantified these structures. Thus, the *Phytophthora* zoospore, in whatever form, has the ability to survive in irrigation water for weeks, a phenomenon which enhances the chance of pathogen dissemination with irrigation water to susceptible crops (Shokes and McCarter, 1979).

Dispersal of *Phytophthora* zoospores in irrigation water. The biflagellate zoospores of *Phytophthora* spp. are able to swim in irrigation water. However,

they have a limited capacity for dispersal through their own active swimming activities unless they are aided by moving water in form of irrigation water or in rainfall runoff (Erwin *et al*., 1983). *Phytophthora* zoospores have been noted to swim in a smooth helical path and can afford a speed of approximately 100-150µm per second (Deacon and Donaldson, 1993). However, this rate of movement is not important because the swimming is interspersed with frequent spontaneous direction changes (Carlile, 1986; Deacon and Donaldson, 1993). Moreover, zoospore movements are often disoriented when they contact surfaces (Erwin and Ribeiro, 1996), causing them to stop swimming and form cysts. The major determinant of zoospore dispersal in aquatic systems must therefore be the water currents, which will also dictate the direction of movement (Neher and Duniway, 1992). They found considerable dispersal of *Phytophthora parasitica* zoospores along the direction of water flow in irrigation furrows. This caused an increase in disease incidence with increasing distance from the water inlet, resulting from zoospores that were transported away from the water inlet to areas of slower current where they settle out. Café-Filho *et al.* (1992b) recorded long distance dispersal of viable propagules of *Phytophthora capsici* and *P. parasitica* downstream with the usual furrow irrigation practices. Their study also found increased accumulation of secondary inoculum with distance and time from the initial inoculum source located upstream. Noticeably, active (autonomous) zoospore dispersal has received comparatively little attention notwithstanding the fact that they must rely on their autonomous swimming activities in water to locate suitable infections sites or substrates. This has led to

little information on the contribution of active/autonomous zoospore swimming on the dispersal of *Phytophthora* spp in water.

The biology of zoospores. The zoospore forms the motile unicellular portion of the life cycle of *Phytophthora* and other oomycetous organisms. They are formed in a zoosporangium by mitotic nuclear divisions and subsequent cleavage by fusion of the vesicles (Hohl and Hamamoto, 1967; Lange and Olson, 1983). The zoospores are discharged through a more or less pronounced papillum (papillation and its degree thereof is species specific), swim for a period, and encyst (Hohl and Mitchell, 1972; Lange and Olson, 1983). The period of motility apparently depends on environmental factors. As the propagules of survival and spread, zoospores undergo a multi-stage sequence before host infection (Deacon and Saxena, 1998). According to Deacon and Donaldson (1993), the motile zoospores seek out and accumulate on a host (usually around the region of elongation immediately behind the root tip), orientate, encyst and adhere on the host surface, before finally germinating to initiate infection.

The zoospore morphology and ultrastructure. The zoospores of *Phytophthora* spp. are typically ovoid, bluntly pointed at one or either ends or reniform (Ho *et al*., 1968a; Lunney and Bland, 1976) and are biflagellate (with anterior tinsel and posterior whiplash flagella). The soma (body) is bound by a unit membrane (Ho and Hickman, 1967) and is longer than it is broad (Carlile, 1983). The biflagellate zoospore of *Phytophthora* has a ventral groove (Ho *et al*., 1968a) that runs the

entire longitudinal axis of the zoospore body. The groove appears deeper at the center (appearing as almost overarched by outgrowths of the zoospore body) than it is at either end. This characteristic shallowness at either end is responsible for the zoospore's reniform shape observed when they are viewed from the anterior (Ho *et al*., 1968a). The two flagella arise from a protuberance in the deep part of the groove region nearer to the anterior than to the posterior. However, the flagella are distinguishable by the presence or absence of lateral appendages (mastigonemes or flimmers) on their surfaces. The tinsel or hispid flagellum has prominent lateral hairs projecting at right angles to the long axis and is oriented anteriorly whereas the posteriorly projected whiplash flagellum is hairless**.**

Ultrastructurally, the zoospore cell protoplasm is bound by a plasma membrane, which extends the entire length of each flagellum (Hoch and Mitchell, 1972; Deacon and Donaldson, 1993). The zoospore has cell organelles such as the ribosomes, mitochondria, endoplasmic reticulum, vesicles, microtubules, and a pyriform shaped nucleus (Ho *et al*., 1968b; Hoch and Mitchell, 1972). The nucleus is bound by a double nuclear membrane and has a large distinct nucleolus. The rough endoplasmic reticulum (RER) occurs as concentric rings around the nucleus. The typical, spherical to oblong or irregular shaped membrane bound mitochondria are abundant in the protoplasm. They contain numerous narrow, elongate cristae lying parallel to one another. The crystalline containing vesicles (Reichle, 1969) are widespread within the cell's cytoplasm, except in the groove region.

The ultrastructural features of the biflagellate zoospore groove region presents an interesting set up with profound effects on the zoospore behavior. The groove region lacks the mitochondria, ribosomes and vesicles. Instead, it has abundant microtubules, a single large contractile vacuole, and several groups of undulating, transversely striated elements of unknown nature (Ho *et al*., 1968b; Hoch, 1972). The groove-associated microtubules are thought to be responsible for mechanical support at the flagella bases. The striated elements are not well understood functionally, but are also presumed to offer further support in this area (Ho *et al*., 1968b). The large central contractile vacuole lying just beneath the groove region seems to aid in osmoregulation.

The flagella originate from protuberances (called kinetosomes or blepharoplasts) in the groove region (Barr, 1983). The two kinetosomes are located in close association with one another. Lunney and Bland (1976) showed that an electron-opaque striated fiber bundle called a rootlet directly connects the two kinetosomes. Other fiber bundles or 'rootlets' partly surround each kinetosome and radiate into the cytoplasm just below the plasmalemma of the zoospore body. The rootlets have 5-8 microtubules. The flagella in cross-section exhibit the typical 9 +2 microtubular formation. Ho *et al*. (1968b) reported that the zoospore axoneme is not different from other flagella. The axoneme consists of a peripheral ring of nine doublet fibrils and two single central fibrils. These are features of importance in zoospore motility.

Zoospore motility and taxes. Biflagellate zoospores of *Phytophthora* species depend on their flagella for detection of external stimuli as well as for motility. Zoospore motility results from the propagation of symmetrical planar sine waves distally (i.e. from base to apex) by both flagella (Cahill *et al*., 1996). In this rhythm, the action of whiplash flagellum pushes the cell through surrounding medium in a direction opposite to that of wave propagation. The action of tinsel flagellum, however, pulls the cell in same direction as that of the wave propagation. The anteriorly directed tinsel flagellum is responsible for most of the thrust, whereas the posteriorly directed whiplash flagellum acts to control direction of movement (Carlile, 1983). The posterior whiplash flagellum is believed to be responsible for the often-observed swift but smooth direction changes in zoospore movements. Deacon and Donaldson (1993) reported that *Phytophthora* zoospores move in a smooth helical path, rotating about their long axis as they swim in an aqueous medium.

The duration of zoospore swimming varies. Thomson (1972), and Thomson and Allen (1974) have recorded zoospore motility extending up to 20 hours for *Phytophthora parasitica* in water. It is not clear whether the observed zoospores were the original released from sporangia or additional released from zoospore cysts. Nevertheless, coupled with a speed of about 100 – 150μm per second (Deacon and Donaldson, 1993), one might expect the zoospores to be able to move long distances through their swimming activities. However, the duration of swimming is affected by such factors as temperature, pH, and the presence or absence of obstacles such as zoospores, shallow suspension

medium, or any other particles interfering with the swimming zoospores (Carlile, 1983). Generally, cool water (10 – 15 C) has been shown to allow the longest swimming period (Carlile, 1986). The motility period can be reduced by such factors as shaking, centrifugation, excessive physical contact, and high carbohydrate concentrations. In addition, the zoospores are known to exhibit numerous direction changes, which also influence their distance of travel. It therefore seems probable that the swimming activities of the zoospore are a means to thoroughly explore a limited volume rather than traveling long distances (Carlile, 1986).

Despite the enormous ecological advantage it confers to *Phytophthora* species, the factors that determine the duration of the motile phase and its termination in irrigation water are poorly understood. Irving and Grant (1984) suggested that naturally occurring pectic materials on the host plant surface provide the necessary recognition and response signals for the commencement of differentiation of the motile zoospore into a cyst. Nevertheless, it is probable that the same factors allow motility to be retained until the cell is within a few millimeters of the host surface and in a locality where, after germination, sufficient nutrition is available to carry it through to the stage where it has access to the contents of living host cells. Low concentrations of divalent cations have also been reported to prolong zoospore motility (Carlile, 1983). A more startling report by Willoughby and Roberts (1994) revealed that physical or ionic shock is able to immobilize *Aphanomyces* zoospores without causing encystment and then resume motility afterwards. This could have considerable implications in

irrigation waters, by ensuring that the zoospores are carried in a quiescent state and only encyst when they approach a suitable substrate or host. The present research studies sought to provide new information on the ability of different generations of zoospores to retain motility in irrigation water.

During motility, zoospores also show various tactic responses to host roots, all of which are essential for the fungal survival (Cameron and Carlile, 1980; Carlile, 1983). The tactic responses include directed movements to chemical gradients (chemotaxis), in water currents (rheotaxis), due to gravity (geotaxis), in electrical fields (electrotaxis), and in response to diffusible factors from other zoospores (autoaggregation) (Khew and Zentmeyer, 1973; Cameron and Carlile, 1977; Cameron and Carlile, 1980; Carlile, 1983; Deacon and Donaldson, 1993; Morris and Gow, 1993). Chemotactic responses help zoospores in locating plant roots by swimming toward regions of nutrient exudation such as the zone of elongation or wounded sites (Khew and Zentmeyer, 1973). Chemotactic responses of zoospores to root exudate compounds like amino acids, sugars and organic acids have been demonstrated *in vitro* (Zentmeyer, 1961; Jones *et al*., 1991). Geotactic and rheotactic responses serve to place the zoospores in the aerobic layers of the soil where roots occur (Carlile, 1983; Cameron and Carlile, 1980). The electrotactic response may act synergistically with chemotaxis in facilitating host location (Ho and Hickman, 1967). Zoospores accumulate at the cathode of an electric field *in vitro*, a factor that may account for the observed electric response toward cathodic root tips (Morris *et al*., 1992).

The phenomenon of formation of zoospore clumps (zoospore autoaggregation) is not well understood, but is thought to result from the attraction of a diffusible factor released by the initial zoospore clumps formed due to random collisions. This mechanism also serves to prevent the formation of further clumps in the vicinity (Carlile, 1983). Whatever the mechanism it could be an important factor in increasing the inoculum potential in these organisms. Often zoospores show taxis to roots and accumulate at the zone of elongation or wounding (Hickman and Ho, 1966).

Zoospore encystment and cyst germination. After a period of active swimming, the duration of which is subject to environmental conditions, the zoospores slow down, assume a sluggish motion, and start to display irregular jerky motions (Lunney and Bland, 1976; Erwin *et al*., 1983). The zoospores retract or shed their flagella, round up, and form double walled spherical structures called cysts (Bartnicki and Wang, 1983). During and before encystment at an infection site, several events take place. First, the zoospores adopt a specific orientation with respect to a potential host before they finally lose their motility. According to Hardham and Gubler (1990), the specific orientation is responsible for the alignment of a predetermined site of germ tube emergence. These workers observed a specific alignment in *Phytophthora cinnamomi* in which the zoospore ventral surface faces the root. This alignment and loss of motility is followed by zoospore cyst secretion of adhesive proteinaceous material, which binds the zoospore cyst to the host surface (Gubler *et al*., 1989)

to prevent any possible dislodgement and to anchor the cyst so the germ tube can physically penetrate the host surface. The encysting zoospore releases Ca^{2+}, which interacts with the adhesive and fixes the cyst to host surface (Deacon and Donaldson, 1993). The released Ca^{2+} is trapped by the adhesive pad which is later reabsorbed to trigger germination.

In nature, zoospore encystment prior to infection is largely a pre-programmed process that only requires a host or substrate trigger (Deacon and Donaldson, 1993). However, agitation, lowering of the pH, or increasing the osmotic potential of a solution or other adverse changes of environmental conditions can hasten zoospore encystment (Hemmes, 1983). Depending on the trigger, different types of encystment have been recognized (Waterhouse, 1962). These are the rapid encystment, which is a prelude to germination and the second type, which occurs presumably to tide over certain adverse conditions. Still, a third type of encystment that accompanies dimorphism (diplanetism) in some fungi does occur. The encystment in response to root surface mucilage polysaccharides has been shown to be a prelude to germination by germ tube (Estrada-Garcia *et al*., 1990). The second and third types of encystment are triggered by unfavorable factors, and may lead to further zoospore emergence or germination failure (von Broembsen and Deacon, 1997; von Broembsen and Charlton, 2000). Studies on the factors that affect zoospore encystment would provide useful information with potential for disease management.

Following encystment and adhesion, a localized bulge on the cyst wall appears (within 30 – 40 min) as a first sign of germination. The bulge initially

develops into a narrow tube (Hoch and Mitchell, 1972), which later expands into assimilative hyphae. Cyst germination is also a very responsive phase and has many triggers. Root exudates (Hickman and Ho, 1966) nutrient mixtures, sugars, amino acids, and several other compounds have been reported to trigger cyst germination (Jones *et al*., 1991; von Broembsen and Deacon, 1997; Deacon and Saxena, 1998). Thus, depending on environmental cues, two possible pathways may follow zoospore encystment. It may germinate by producing a germ tube or by releasing a further zoospore, a cycle which may occur repeatedly. The production of further zoospore from the cysts is thought to be a "default" option (Deacon, 1996; von Broembsen and Deacon, 1997) adopted in case of failure by these parasites to locate a suitable host or substrate after swimming for a time.

There is little information on the precise factors that promote or suppress cyst germination *in vivo*. It is not always clear whether factors that induce cyst formation could suppress or promote subsequent cyst germination. Carlile (1983) postulated that factors that signal favorable conditions (like root exudates) are likely to stimulate both encystment and subsequent germination, but unfavorable factors (like physical or chemical shock) that stimulate encystment may inhibit germination by germ tubes but promote further zoospore emergence. Zoospore encystment induced by basic amino acids and Fe^{3+} within certain concentration ranges produced cells less committed germinate (Byrt *et al*., 1982). Tellingly, other compounds such as simple sugars or acidic amino acids are essential at the time of the reception of encystment stimulus for the cell to proceed to germ

tube formation. These germination-inducing compounds do not have the capacity to induce encystment on their own (Byrt *et al*., 1982). These findings undermine the theory that the cell transformation from the motile state to the germination state depends on a cascade effect in which one single factor triggers the pre-infection process. It is probable that zoospore germination near host surfaces results from exposure to multiple germination cues.

Zoospore repeated emergence (re-emergence). It is generally assumed that under the normal course of events, zoospores usually settle down after a swarm period, encyst, and germinate by a germ tube. However, a number of cases where zoospores encyst in water and re-emerge for another swimming period have been reported for *Dictyuchus* (Weston, 1919), *Achlya* (Salvin, 1940), *Aphanomyces* (Cerenius and Söderhäll, 1984, 1985; Lilley *et al*., 1999), *Saprolegnia* (Diéguez-Uribeondo *et al*., 1994), *Pythium* (Jones *et al*., 1991) *Phytophthora* (von Broembsen and Deacon, 1997), *Phytophthora sojae* (Xu and Morris, 1998) and several other *Phytophthora* spp. (von Broembsen and Charlton, 2000). This phenomenon is properly called “repeated emergence” in *Phytophthora* (Blackwell, 1949) as opposed to diplanetism, which should mean diplanetism with dimorphism. Diplanetism is yet to be reported for *Phytophthora* spp. In diplanetism, the zoospore when it first emerges from the sporangia is pear shaped with two terminal flagella, but after second emergence, and later ones, is bean shaped with two lateral flagella (Blackwell, 1949; Alexopoulos *et al*., 1996). The term repeated emergence has also been used for the later

swarming stages in *Dictyuchus* (Weston, 1919), *Saprolegnia* (Diéguez-Uribeondo *et al*., 1994), and is therefore appropriately used in *Phytophthora* and *Pythium* where only the laterally biflagellate zoospore is known.

Repeated zoospore emergence is thought to be an adaptive feature (Cerenius and Söderhäll, 1985) which functions to prolong the motile phase, thereby providing the parasite an opportunity to further explore its environment in case a suitable substrate or host is not immediately located (Ho and Hickman, 1967). It is also thought to offer a "rejuvenation" opportunity by ushering in a period of quiescence for a certain mass of protoplasm, which later emerges as a thoroughly revived entity (Salvin, 1940). Repeated emergence has been reported to be responsive to environmental cues in nature. Xu and Morris (1998), observed the suppression of repeated emergence of zoospores in *Phytophthora megasperma* var. *sojae* by root substances and nutrient solutions. von Broembsen and Deacon (1997) also found that Ca^{2+} triggers zoospore cyst germination in *Phytophthora parasitica* but suppresses further zoospore release from ungerminated cysts.

Despite the reported cases of repeated zoospore emergence in several Pythiaceous fungi including *Phytophthora sojae* (Ho and Hickman, 1967), *Pythium aphanidermatum* (Jones *et al*., 1991), and *Phytophthora parasitica* (von Broembsen and Deacon, 1997), its extent and prevalence remains to be reported among this group of pathogens. Moreover, there is reason to believe that the extent of repeated zoospore emergence is a variable character among fungal species. This is because zoospores are dependent on stored energy and thus

on reserve food material (Salvin, 1940). It has also been speculated that the repeated emergence of zoospores from the encysted state is under the control of some inherent factor (Salvin, 1940). This is an interesting phenomenon worthy of further investigation.

Zoospores that emerge from cysts during repeated emergence are structurally similar to those released from the sporangia (sporangial derived zoospores) (Xu and Morris, 1998). Salvin (1940) recorded up to five successive swarm stages of motile *Achyla* zoospores, which had similar shape but with decreased amount of material with each generation. While working with *Saprolegnia parasitica,* Diéguez-Uribeondo *et al.* (1994) also reported a record six swarm stages. The ability of these cysts to produce additional generations of zoospores appeared to be related to stored reserves. If this were true, then one would expect the reserves to decline with each successive generation. This would subsequently render the cyst derived zoospores less biologically fit than the sporangia derived zoospores. However, in no case has a comparative study on any component of biological fitness between these two types of zoospores been attempted for any fungus. This study explored the ability of both sporangial and cyst derived zoospores to disperse autonomously, to locate roots in water, and to retain motility.

RESEARCH JUSTIFICATION

Phytophthora spp. are destructive plant pathogens with complex asexual life cycle stages that produce various infectious propagules which include

mycelium, sporangia, zoospores and zoospore cysts (de Souza *et al*., 2003). Each of these stages is under the influence of different environmental factors such as temperature, water relations, physical and chemical conditions, and with interacting combinations of these factors (Fawcett, 1936; Zentmeyer, 1981; Duniway, 1983). Therefore, the geographic distribution of *Phytophthora* spp. and the diseases they cause depends on the constraints of any of these environmental factors on any or all of the life cycle stages (Duniway, 1983). Of the environmental factors, temperature has been reported to have a large influence on growth, reproduction and pathogenesis of *Phytophthora* spp. (Sujkowski, 1987; Sing and Chauhan, 1988; Matheron and Matejka, 1992). However, most of these studies take into account only one species or isolate of *Phytophthora* and consider only one particular stage in the life cycle (Gooding and Lucas, 1959; Zentmeyer and Erwin, 1970; Zentmeyer, 1981; Sujkowski, 1987; Matheron and Matejka, 1992; Sato, 1994; Roy, 1999; Timmer *et al.*, 2000). The current study examined the effect of temperature on the asexual life cycle stages which included the mycelial growth, sporangia production, and zoospore cyst germination of several water-borne *Phytophthora* spp. An understanding of the influence of temperature on these important life cycle developmental stages is essential step in the development of best management practices for these pathogens in irrigation water.

Phytophthora zoospore cysts, depending on environmental cues have different options. They have the ability to remain encysted, germinate by germ tubes, or by releasing additional zoospores (repeated emergence) (von

Broembsen and Deacon, 1997; Xu and Morris, 1998; von Broembsen and Charlton, 2000). The zoospores that emerge from the cysts during re-emergence are structurally similar to zoospores released from the sporangia. Repeated zoospore emergence is an adaptation to parasitism with great value for survival and spread for plant pathogenic oomycetes, which exhibit it (Cerenius and Söderhäll, 1985). The intervening encystments probably function to offer the zoospore an opportunity to replace worn out flagella and to further provide opportunity for zoospore to disperse and thoroughly explore its environment. This phenomenon is also thought to offer a "rejuvenation" opportunity by ushering in a period of quiescence for a certain mass of protoplasm, which later emerges as a thoroughly revived entity (Salvin, 1940). Despite the great survival value this intriguing phenomenon is thought to confer, it remains poorly understood in *Phytophthora* spp. The current study sought to characterize and document the occurrence of this phenomenon and the possible role of temperature as an important environmental cue which influences repeated zoospore emergence among *Phytophthora* spp. common in irrigation water. Understanding this phenomenon is an essential step in understanding the basic biology and epidemiology of water-borne *Phytophthora* spp.

Zoospore motility and autonomous dispersal are important pre-infection stages, which must be accomplished with precision if successful host infection is to occur (Donaldson and Deacon, 1993). The motile phase aids in dispersal of *Phytophthora* by increasing the probability of encountering a new host. Prolonged motility could thus have considerable epidemiological implications for

these pathogens in irrigation water. Zoospores depend entirely on endogenous energy reserves and only acquire the competence to take up exogenous energy sources upon germination (Pennington *et al*., 1989). Thus, even though zoospores that emerge from cysts are similar structurally to those released from sporangia (Xu and Morris, 1998), they may have their energy reserves severely depleted with each successive swarm stage. It is therefore probable that the biological fitness of subsequent zoospore generations become limited. However, there is currently no information comparing important components of biological fitness of zoospore released from sporangia with those that emerge from cysts for *Phytophthora* spp. This study compared autonomous dispersal, root location and infection, and motility retention for these zoospore types for several *Phytophthora* spp. Such studies are essential in understanding the ecological role of cyst derived zoospores and consequently formulating relevant *Phytophthora* disease management support systems in irrigated cropping systems.

RESEARCH OBJECTIVES

The study specific objectives were:

1. To investigate, characterize, and document the effect of temperature on asexual life cycle stages and repeated zoospore emergence (swarming) by several *Phytophthora* spp. common in irrigation water.
2. To compare autonomous dispersal, and root location and infection by of sporangial and cyst derived zoospores of several *Phytophthora* spp. common in irrigation water.
3. To compare the ability of sporangial and cyst derived zoospores to retain motility under different temperature regimes.

CHAPTER III

EFFECT OF TEMPERATURE ON *PHYTOPHTHORA* MYCELIAL GROWTH, SPORANGIA PRODUCTION, AND ZOOSPORE REPEATED EMERGENCE

Phytophthora spp. are ubiquitous pathogens of a number of important crops worldwide (Erwin *et al*., 1983; Erwin and Ribeiro, 1996; Agrios, 1997) and possess a high level of diversity with over 67 species described (Erwin and Ribeiro, 1996). These pathogens have a complex asexual life cycle with distinct multiple infectious propagules which include the mycelium, sporangia, zoospores, and zoospore cysts (de Souza *et al*., 2003). *Phytophthora* pathogenesis is influenced by different environmental factors such as temperature, water relations, physical and chemical conditions, and with interacting combinations of these factors (Fawcett, 1936; Zentmeyer, 1981; Duniway, 1983). The geographic distribution and seasonality of *Phytophthora* diseases depends on the constraints of any of these environmental factors on any or all of the life cycle stages (Duniway, 1983). Of the environmental factors, temperature has been reported to have greatest influence on growth, reproduction and pathogenesis of *Phytophthora* spp. (Sujkowski, 1987; Sing and Chauhan, 1988; Matheron and Matejka, 1992). Effects of temperature on soilborne *Phytophthora* spp. have been reported (Zentmeyer and Erwin, 1970; Zentmeyer, 1981; Sujkowski, 1987; Matheron and Matejka, 1992). However,

most of these studies take into account only one species or isolate of *Phytophthora* and consider only one particular stage in the life cycle (Gooding and Lucas, 1959; Zentmeyer and Erwin, 1970; Zentmeyer, 1981; Sujkowski, 1987; Matheron and Matejka, 1992; Sato, 1994; Roy, 1999; Timmer *et al.*, 2000).

Mycelial development by *Phytophthora* spp. is an important component of disease development (Zentmeyer, 1981; Simpfendorfer *et al.*, 2001). The temperature in any given environment must be permissive to mycelial development and growth for the respective *Phytophthora* spp. to support disease progress (Duniway, 1983). The parallel effects of temperature on mycelial growth and the development of *Phytophthora* diseases have been described (Fawcett, 1936; Zentmeyer *et al.*, 1976; Zentmeyer, 1981; Duniway, 1983; Matheron and Matejka, 1992). Zentmeyer (1981) reported a temperature correlation between *P. cinnamomi* mycelial growth and *Phytophthora* root rot disease development of avocado. However, such results should be interpreted with caution. Matheron and Matejka (1992) reported a lack of correlation between *Phytophthora parasitica* and *P. citrophthora* mycelial growth on corn meal agar (CMA) and disease development. This could be due to the fact that temperature affects only the physiology of the pathogen when grown *in vitro*. Alternatively, *Phytophthora* disease development is the resultant, cumulative effect of temperature on the physiological processes of both the pathogen and the host.

Even though *Phytophthora* growth *in vitro* at various temperatures may offer limited predictive value of disease development on plant tissues at the same temperatures, understanding the influence of temperature on the *in vitro* mycelial

development of *Phytophthora* spp. is an essential step in the development of best management practices for these pathogens in irrigated agriculture.

Sporangia production is an important stage in the life cycle of *Phytophthora* spp., as it provides the opportunity for rapid increase in the numbers of infective propagules and disease (Matheron and Matejka, 1992). Sporangia usually germinate either indirectly by releasing zoospores or directly by growing germ tubes and thus increasing plant tissue infection (Gooding and Lucas, 1959; Matheron and Matejka, 1992; Sato, 1994; Timmer *et al*., 2000). The influence of temperature on the rate of sporangia formation in *Phytophthora* spp. has been documented (Gooding and Lucas, 1959; Duniway, 1983; Matheron and Matejka, 1992; Sato, 1994; Timmer *et al*., 2000). In most *Phytophthora* spp., the temperature range over which sporangia can form appears to be narrow and this is thought to confine their activities to certain seasons of the year (Fawcett, 1939; Duniway, 1983). Usually, significant *in vitro* sporangial production occurs within a wide range of temperature whereas the optimal range is quite narrow for a given *Phytophthora* spp. Studies on the influence of temperature on sporangial production by water-borne *Phytophthora* spp. is an important prerequisite for developing disease predictive models in the field.

Zoospores are primary propagules of infection by oomycetes, including *Phytophthora* spp., especially under wet conditions and in irrigated cropping systems. The infection process begins with zoospore orientation towards a potential host, their encystment and adherence to a host surface, and their subsequent germination (Hardham and Gubler, 1990). In this scenario,

encystment is largely a pre-programmed process that only requires a host or substrate trigger (Deacon and Donaldson, 1993). Environmental cues such as physical agitation, heat or chemical shock, lowering of the pH, or increasing the osmotic potential of a solution have been noted to hasten zoospore encystment (Ho and Hickman, 1967; Hemmes, 1983; Willoughby and Roberts, 1994). After a period of active swimming without locating a host or substrate *Phytophthora* zoospores slow down, assume a sluggish motion, and start to display irregular jerky motions (Lunney and Bland, 1976; Erwin *et al*., 1983). They shed their flagella, round up, and form double walled spherical structures (Bartnicki-Garcia and Wang, 1983) called cysts. Depending on environmental cues, encysted zoospores may remain encysted, germinate by germ tubes or by releasing additional zoospores (repeated emergence).

Repeated zoospore emergence has been observed in both sporangial and cyst derived zoospore cysts (von Broembsen and Charlton, 2000) of water-borne *Phytophthora* spp. This phenomenon is thought to be an adaptive feature which offers great opportunity for survival and spread for plant pathogenic oomycetes which exhibit it (Cerenius and Söderhäll, 1985). The intervening encystments probably function to offer the zoospore an opportunity to replace worn out flagella, and provide further opportunity to thoroughly explore its environment, and offer to a “rejuvenation” period of quiescence (Salvin, 1940). Despite great survival value and epidemiological implications of this phenomenon, little is known about its occurrence and prevalence for water-borne *Phytophthora* spp.

Understanding this phenomenon is an essential step in the formulation of effective management practices of water-borne *Phytophthora* spp.

The objective of this study was to investigate the occurrence and prevalence of zoospore repeated emergence (re-emergence) among *Phytophthora* spp. common in irrigation water. The ability of both sporangial and cyst derived zoospore cysts to germinate by further zoospore release was investigated for various *Phytophthora* spp. that are commonly associated with irrigated cropping systems. The role of temperature as a possible environmental factor that influences this process was also evaluated.

MATERIALS AND METHODS

Media. Clarified V8 broth was prepared by mixing 340 ml of V8 juice (Campbell Soup Co., Camden, NJ) with 5 g $CaCo_3$ and stirred with a magnetic stirrer for 30 min. The mixture was then centrifuged at 10,000 rpm for 10 min, the supernatant decanted and diluted (1:5, v/v) with sterile distilled water (SDW). The broth was dispensed into bottles (200 ml per 500 ml Pyrex bottle) before autoclaving for 15 min at 121^{o}C (von Broembsen and Deacon, 1996). A clarified V8 agar was prepared by adding 15 g of Difco Bacto Agar (Difco Laboratories, Detroit, Michigan) to 1L V8 broth and autoclaving again (Ribeiro, 1978). The mineral salts solution comprised of $Ca(NO_3)_2.4H_2O$ (3.08 g), $MgSO_4.7H_2O$ (1.49 g), and KNO_3 (0.51 g) per liter of distilled water (von Broembsen and Deacon, 1996). The solution was autoclaved and 1 ml of filter sterilized chelated iron solution added.

The iron solution comprised of ethylenediamine-tetraacetic acid (EDTA) (0.6525 g), KOH (0.375 g), and $FeSO_4.7H_2O$ (1.245 g) in 50 ml sterile distilled water (SDW).

Experimental cultures. Isolates of *Phytophthora parasitica* (Brenda de Haan) Dastur (GLN 9-3), *P. citrophthora* (Sm. *et* Sm.) Leonian (GLN 7-23), *P. citricola* Sawada (PHP R-2), and *P. cinnamomi* Rands (1D-A) originally isolated from irrigation water or irrigated nursery crops in Oklahoma and maintained on malt extract agar (MEA; 10 g difco malt extract and 15 g difco bacto agar per liter of distilled water) were used in the experiments described. *P. cryptogea* (Perthybridge and Lafferty) isolates FDM51 and FWDM4 from dusty miller (*Senecio cineraria*) were supplied by Dr. M. Benson of North Carolina State University, Raleigh, NC.

Effect of temperature on mycelial mat development. The effect of temperature on mycelial mat growth of *Phytophthora parasitica* (GLN 9-3), *P. citricola* (PHP R-2), *P. citrophthora* (GLN 7-23), and *P. cryptogea* (isolates FDM51 and FWDM4) was assessed as follows. Each isolate was grown on V8 agar for 4 days and mycelial disks (5 mm diameter) from actively growing colony margins were cut with a sterile cork borer and distributed (3 disks per petri dish) into 65 mm petri plates each containing 3 ml of clarified V8 broth. The plates were then incubated at 15, 20, 25, or 30 C and colony diameter measurements taken at 24, 48, and 72 h by taking two perpendicular readings of each colony and subtracting the diameter of the inoculation disk. A total of two replicate plates

were inoculated for each isolate and temperature combination and the experiment was repeated once.

Effect of temperature on sporangium production. Each isolate was grown on V8 agar in 65 mm petri plates. After 4 days, 5 mm agar disks were obtained from actively growing colony margins and distributed (3 disks per plate) into 65 mm petri plates each containing 3 ml of mineral salt solution (MSS). The plates were incubated at 15, 20, 25, or 30 C in the dark for 3 - 4 days after which, the numbers of sporangia along the periphery of four arbitrarily selected fields of view for each disk were counted under the microscope at a magnification of X100. There were two replicate plates for each isolate and temperature and the experiment was repeated three times.

Zoospore production. Zoospores were produced axenically from each *Phytophthora* isolate following a protocol previously described by von Broembsen and Deacon (1997). Prior to use, the cultures were plated onto cornmeal agar {CMA; 17 g cornmeal agar per liter of distilled water (Sigma-Aldrich, St. Louis, MO)} and incubated at 25 C for 4 - 5 days. Cultures were then transferred to clarified V8 agar and incubated at 25 C for 3 - 4 days. Agar blocks (5 x 5 mm) were cut with a sterile scalpel from colony margins and distributed (10 per plate) into 90 mm petri plates each containing 10 ml clarified V8 broth. The plates were incubated for 36 h (for *P. cryptogea* isolates), or 24 h for all other isolates at 25 C to allow mycelial mats to develop.

Following incubation, the broth from each plate was removed and the mats washed three times with mineral salts solution (MSS) as follows: 10 ml of MSS was added into each plate and the plates swirled gently to wash the mats. At the end of the third washing the plates were flooded with 10 ml of MSS and incubated at 20/21 C beneath (~ 16 cm) fluorescent lights. After 24 h, the MSS from each plate was removed, replaced with additional 10 ml of MSS, and returned to incubation under same conditions. After 4 days of further incubation, the MSS was decanted, the mats were rinsed twice with sterile distilled water (SDW), each plate flooded with 10 ml of SDW, and immediately placed in a refrigerator (4 C) for 45 - 50 min to stimulate cleavage of sporangium contents to form zoospores (von Broembsen and Deacon, 1997). After 45 – 50 min, the plates were removed from the refrigerator and placed on bench racks at 21 – 22 C to allow zoospore release.

Zoospore suspensions were harvested from each plate after 1 - 2 h by tilting and pipetting the top 8 ml into McCartney bottles (16 ml per bottle for a total of 5 bottles). Zoospores are negatively geotropic (Cameron and Carlile, 1977; Charlton, 2001) and tend to concentrate at the upper third of liquid in tilted plates (von Broembsen and Deacon, 1997).

Induction of zoospore encystment. Zoospore suspensions were encysted by holding each McCartney bottle against a Maxi Mix II vortex machine (Whatman Inc., Clifton, NJ) for 2 min. The cyst suspensions from all bottles were

immediately mixed together in a sterile 100 ml Pyrex bottle and gently swirled to homogenize the suspension.

Experimental design. Sporangial derived zoospore **(**SDZ) cysts produced as described above were incubated at 15, 20, 25, or 30 C in Eppendorf tubes or McCartney bottles for 7 – 8 h. For every incubation temperature, four replicate Eppendorf tubes (each containing 1 ml of cyst suspension) and a McCartney bottle containing 16 ml of cyst suspension were prepared. The four Eppendorf tubes were used to assess the mode of SDZ cyst germination as described below. Previous studies by Charlton and von Broembsen (2001) established that zoospore re-emergence from cysts occurs by 7 – 8 h after vortex encystment, and the released zoospores tend to congregate near the upper surface of the liquid (Cameron and Carlile, 1977; von Broembsen and Deacon, 1997; Charlton, 2001). At 7 – 8 h, cyst derived zoospore suspensions were obtained by removing the upper third of liquid from each McCartney bottle and dispensing into four Eppendorf tubes (each Eppendorf tube received 1 ml aliquot of zoospore suspension). Each Eppendorf tube was held against a vortex mixer for 70 sec (to produce CDZ cysts) and returned to incubation at their respective temperatures for a further 6 - 7 h. Four replicate Eppendorf tubes were prepared for each treatment and each experiment was repeated three times.

Assessment of germination of SDZ cysts. After 7 – 8 h of incubation, each Eppendorf tube was briefly held against a vortex mixer for 3 sec to resuspend the

contents and then 80 µl was spread on precleaned plain 3 x 1 inch microscope slides (Fisher Scientific Co., Pittsburg, PA). The slides were supported underneath by glass rods in 150 x 120 mm pyrex petri-dishes lined with moist paper towels to maintain wetness prior to mode of germination assessment. The mode of germination was assessed for 100 cells per slide microscopically at 200X using a photomicrographic attachment (Nikon Corp., Chiyoda-Ku, Tokyo) and a video screen. The cells were scored as ungerminated, germinated by germ tube, or germinated by a single zoospore release (von Broembsen and Deacon, 1997). Cells were considered to have germinated by germ tubes if the germ tube length exceeded twice the cell's diameter. Germination by further zoospore release (re-emergence or repeated emergence) was represented by the presence of empty cyst cell walls (ghosts) with or without a short exit tube (Figure 3.1).

Assessment of germination of sporangial derived zoospore (CDZ) cysts. At 6 - 7 h post-encystment of CDZs, 80 µl aliquots were obtained from each Eppendorf tube, spread on microscope slides and treated as described previously. The mode of CDZ cyst germination was assessed for 100 cells on each slide as described above for SDZ cysts.

Data analysis. The experiment with each isolate was repeated three times with four replications per treatment (temperature). Since there were no trial effects,

data for each isolate were pooled and analyzed using the PROC GLM (SAS Institute Inc., Cary, NC, 2001).

RESULTS

Effect of temperature on mycelial mat growth. All five *Phytophthora* isolates tested in this study were able to survive and attain varying degrees of mycelial growth from 15 – 30 C (Table 3.1). After 72 h, *Phytophthora parasitica* (GLN 9-3) had greater ($P \leq 0.05$) mycelial growth at 25 and 30 C, whereas *P*. *citrophthora* (GLN 7-23) had greater growth at 20 C. *Phytophthora citricola* (PHP R-2) showed greater growth at 15 and 20 C after 72 h, and *P. cryptogea* isolates FDM51 and FWDM4 had greatest growth at 20 C and at 20 and 25 C respectively. Discernable variations existed in isolate mycelial growth rates at the different temperatures tested (Table 3.2). *Phytophthora citrophthora* (GLN 7-23) had the greatest ($P \leq 0.05$) growth rate at 20 C and P. citricola (PHP R-2) at 15 and 20 C, whereas *P. cryptogea* isolates FDM51 and FWDM4 had higher growth rates at 15 C and at 20 and 25 C, respectively.

Effect of temperature on sporangial production. Appreciable sporangial production by all *Phytophthora* isolates in mineral salt solution (MSS) occurred at 25 C (Table 3.3). However, considerable variation in the extent of sporangial production was observed between the isolates at different temperatures. *Phytophthora citrophthora* (GLN 7-23) and *P. cryptogea* (FDM51) both had greater ($P \leq 0.05$) sporangial production at 25 and 30 C, whereas *P. cryptogea*

(FWDM4) had the most prolific sporangial formation at 30 C. *Phytophthora citricola* (PHP R-2) had the greatest sporangial production at 25 C, whereas *P. parasitica* (GLN 9-3) had the greatest sporulation at 25 C. *Phytophthora citricola* (PHP R-2) produced no sporangia at 30 C, and *P. citrophthora* (GLN 7-23) was the most prolific sporangial producer (optimum 25 and 30 C).

***Phytophthora* zoospore cyst germination.** Cysts of *P. citrophthora, P. citricola, P. parasitica,* and *P. cryptogea* germinated by either producing a further zoospore (repeated emergence), by a germ tube, or remained ungerminated in sterile distilled water (SDW). On rare occasions (<1%), cysts germinated by issuing short germ tubes, which developed miniature sporangia at their tips, releasing a single zoospore. The mode of germination was apparently influenced by temperature. Therefore the influence of water temperature on mode of sporangial and cyst derived zoospore cyst germination was further investigated for select *Phytophthora* isolates that were prolific producers in our preliminary studies, namely; *P. citrophthora* (GLN 7-23), *P. citricola* (PHP R-2), *P. parasitica* (GLN 9-3), and *P. cryptogea* (isolates FDM51 and FWDM4).

Effect of temperature on SDZ cyst germination. Temperature influenced the mode of SDZ cyst germination differently for each isolate considered in this study (Table 3.4). Cyst germination by repeated emergence (re-emergence) was higher ($P \leq 0.05$) for *P. cryptogea* (FWDM4) at 20 and 25 C and optimum for *P. cryptogea* (FDM51) at 30 C. *Phytophthora parasitica* (GLN 9-3) exhibited cyst

germination by re-emergence at 20, 20, 25 and 30 C (optimum at 20 C). Re-emergence for these cysts in *P. citrophthora* (GLN 7-23) and *P. citricola* (PHP R-2) were low and did not show any differences across all temperatures considered in this study. Conversely, cyst germination by germ tubes was low ($P \leq 0.05$) for *P. cryptogea* (FDM51 and FWDM4) and *P. citricola* (PHP R-2) across all temperatures. This mode of germination was greater (up to 68%) for *P. parasitica* (GLN 9-3) at 25 C and across all temperatures for *P. citrophthora* (GLN 7-23). Some cysts remained ungerminated in each case, but were not followed to determine their final fates in this study.

Effect of temperature on CDZ cyst germination. The mode of cyst germination for each isolate was also influenced differently by temperature (Table 3.5). *Phytophthora cryptogea* (FWDM4) cysts had higher ($P \leq 0.05$) germination by re-emergence at 20 and 25 C than at 30 C, whereas, *P. cryptogea* (FDM51) had higher germination by re-emergence at 30 C. This mode of germination for *P. parasitica* (GLN 9-3) was greater at 20 C than at 15 and 30 C, for *P. citricola* (PHP R-2) at 20, and for *P. citrophthora* (GLN 7-23) at 20 and 25 C. Cyst germination by germ tubes was consistently low (<13%) for *P. cryptogea* (isolates FDM51 and FWDM4) and *P. citricola* (PHP R-2) at all temperatures. *phytophthora citrophthora* (GLN 7-23) had high germination by germ tubes across all temperatures with a maximum of 69% at 30 C, whereas *P. parasitica* (GLN 9-3) germination by germ tubes was optimum at 25 and 30 C. There was a

tendency for greater percent germination by re-emergence for CDZs than for SDZs for all isolates in this study.

DISCUSSION

The life cycle of *Phytophthora* spp. consists of several distinct asexual developmental stages that are each crucial for plant infection and disease development (Hardham, 2001). These include vegetative growth of filamentous hyphae, sporulation, release of motile zoospores, zoospore encystment, and cyst germination which eventually initiate plant infection (Kennedy and Pegg, 1990). The results of this study demonstrated that temperature is an important environmental factor with differential effects on growth stage and could have important implications for disease and disease management.

The results obtained in this study confirmed the influence of temperature on mycelial mat growth for different *Phytophthora* spp. The influence of temperature on mycelial growth of different *Phytophthora* spp. have been reported (Waterhouse and Blackwell, 1963; Zentmeyer *et al*., 1976; Zentmeyer, 1981; Sujkowski, 1987; Roy, 1999; Simpfendorfer *et al.*, 2001; Dirac and Menge, 2002). Waterhouse and Blackwell (1963) reported an optimum mycelial growth temperature of 25 – 28 C for *P. citricola,* while *P. cryptogea* was observed to have optimum mycelial growth at 20 – 25 C (Kennedy and Pegg, 1990), *P. citrophthora* at 26 C (Dirac and Menge, 2002) and *P. parasitica* at 30 – 31 C for (Matheron and Matejka, 1992; Dirac and Menge, 2002). Results obtained in the *in vitro* studies with water-borne isolates of these species were in general

agreement with these reports. The slight variations observed in our study could be due to the effect of growth media composition, pH and inherent differences between isolates of each species. The influence of nutrient composition on the *in vitro* mycelial growth of *Phytophthora* spp. has previously been reported (Zentmeyer *et al*., 1976; Matheron and Matejka, 1992). The pH of artificial media has also been reported (Wong *et al*., 1986; Simpfendorfer *et al*., 2001) to influence mycelial growth *in vitro*.

Sporangia production is an important stage in the life cycle of *Phytophthora* spp., providing the opportunity for increase in infective units and increased disease (Gregory, 1983; Matheron and Matejka, 1992). *Phytophthora* sporangia can germinate either indirectly by releasing zoospores or directly by growing germ tubes and thus enhancing plant infection (Gooding and Lucas, 1959; Matheron and Matejka, 1992; Sato, 1994; Timmer *et al*., 2000). Results obtained in our studies show that sporangia production in *Phytophthora* is influenced differently by temperature. Production of sporangia in soil by *Phytophthora* spp. has been documented (Duniway, 1983). The temperature response variability among related genera of *Phytophthora* has also been reported (Gooding and Lucas, 1959). Our results on the effect of temperature on *Phytophthora* sporangia production very closely follow those previously reported (Gooding and Lucas, 1959; Matheron and Matejka, 1992; Mizubuti and Fry, 1998; Timmer *et al*., 2000). The temperature range over which the tested isolates were able to produce abundant sporangia appeared to be narrow, suggesting

that temperature as a factor may confine the pathogenic activities of *Phytophthora* to certain geographic locations and seasons of the year.

The observation that cysts of waterborne *Phytophthora* spp. can remain ungerminated, or germinate either by germ tubes or by further zoospore release following vortex-induced encystment is a feature shared with several other spp. of oomycetes including *Dictyuchus* (Weston, 1919), *Achyla* (Salvin, 1940), *Phytophthora megasperma* var. *sojae* (Ho and Hickman, 1967), *Aphanomyces* (Cerenius and Söderhäll, 1985), *Pythium aphanidermatum* (Jones *et al*, 1991), *Saprolegnia parasitica* (Diéguez-Uribeondo *et al*., 1994), *P. sojae* (Xu and Morris, 1998) and *Phytophthora parasitica* (von Broembsen and Deacon, 1997; von Broembsen and Charlton, 2000). The observed ungerminated cysts probably were still in a quiescent state waiting to germinate later. However, their final fates were not studied.

Both SDZ and CDZ cysts exhibited germination by re-emergence, but re-emergence was greater for the latter cyst type. The reason for the observed enhanced germination by re-emergence for CDZs is not clearly known, but this could be because we selected for re-emergence competent cells in each case as we harvested the cyst derived zoospores. For clarity of terminology, we have chosen to use the terms “repeated emergence”, “re-emergence” or “further zoospore release” to describe this phenomenon as opposed to diplanetism, which would include dimorphism (Blackwell, 1949). Diplanetism has yet to be reported for *Phytophthora* spp. The zoospores that emerge from cysts are structurally similar to zoospores released from sporangia. During re-emergence,

the released zoospore wiggles its way out of the cyst, leaving the cyst membrane (ghost) behind with or without a prominent exit tube as described by Ho and Hickman (1967) for *Phytophthora megasperma.*

The ecological significance, if any, of repeated emergence in natural conditions is still a matter of speculation, but the fact that *Phytophthora* spp. appears to dedicate some of their limited endogenous energy to produce precursors or other materials needed for additional generations of zoospores suggests that repeated emergence may play a role *in vivo*. Also, repeated emergence of zoospores could have implications as a survival strategy in the absence of hosts by allowing cysts to delay germination until more favorable circumstances and then releasing further zoospores to achieve infection (Cerenius and Söderhäll, 1985; Diéguez-Uribeondo *et al*., 1994; von Broembsen and Deacon, 1997).

Results obtained in this study is in agreement with previous observations by von Broembsen and Charlton (2000) on the ability of both sporangial and cyst derived cysts of water-bone *Phytophthora* spp. to germinate by repeated emergence (re-emergence). Additionally, re-emergence was observed for a fifth species, *P. cryptogea* (isolates FDM51 and FWDM4), and this suggests that re-emergence could be common among members of the genus *Phytophthora.* Cysts that germinate by germ tubes may later produce microsporangia (<1%) but a vast majority are probably not important in a hostile environment such as irrigation water devoid of a suitable substrate to support further mycelial growth. Our findings clearly demonstrate that temperature is an external signal that has a

profound impact on the mode of germination for both SDZ and CDZ cysts of *Phytophthora* spp. To our knowledge, this is the first report on the effect of temperature on re-emergence from cysts of second and third generations of *Phytophthora* zoospores.

This study provides data with implications for the different aspects of *Phytophthora* disease epidemics in wet and irrigated agriculture. Collectively, the results suggest that the different stages of water-borne *Phytophthora* spp. that are part of the asexual life cycle differ considerably in their response to temperature. Mycelial growth and zoospore cyst germination were the least sensitive to temperature differences and sporangial production the most sensitive. The differences of isolates in response to temperature need to be considered when developing *Phytophthora* disease management support decision systems in the future. Also, this study provides additional experimental data supporting the observations of Ho and Hickman (1967), von Broembsen and Deacon (1997), Xu and Morris (1998), von Broembsen and Charlton (2000) that *Phytophthora* spp. are able to undergo repeated emergence in water.

SUMMARY AND CONCLUSIONS

1. Several asexual developmental life cycle stages of *Phytophthora* spp. (mycelial growth, sporangia production, and zoospore repeated emergence) are influenced differently by temperature, a fact that may

account for distribution of *Phytophthora* diseases or their upsurge during certain seasons of the year.

2. Vortex-encysted *Phytophthora* zoospores are able to germinate in SDW either by re-emergence, or germ tubes. The subsequent generations of zoospores are structurally similar to the initial biflagellate zoospores released from the sporangia.
3. Re-emergence is a common mode of cyst germination for five different water-borne *Phytophthora* spp.
4. The effect of temperature on the mode of *Phytophthora* cyst germination varies with isolates of each species.

Figure 3.1 Ghosts of zoospore cysts (arrows) after germination by release of a single zoospore (re-emergence)

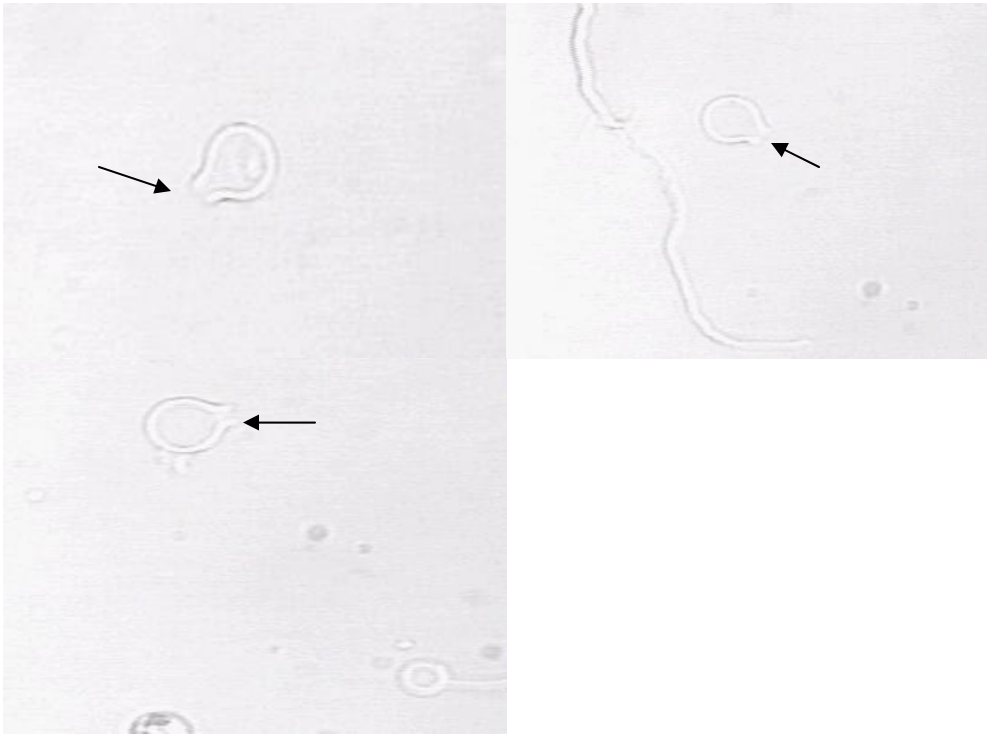


Table 3.1 Effect of temperature on mean colony diameter of five *Phyophthora* isolates in V8 broth after 24, 48, and 72 h

		Mean colony diameter (mm)[a]		
Isolate	**Temp (C)**	**24 h**	**48 h**	**72 h**
P. citrophthora (GLN 7-23)	15	5.3C[b]	11.0B	16.8B
	20	7.1AB	13.7A	20.1A
	25	7.8A	13.1A	16.2B
	30	6.7B	12.9A	17.5B
	LSD	0.88	1.31	2.18
P. parasitica (GLN 9-3)	15	0.7C	3.3C	4.6C
	20	2.8B	6.0B	10.3B
	25	5.6A	10.9A	15.4A
	30	6.1A	11. 6A	14.8A
	LSD	0.54	0.73	1.26
P. cryptogea (FDM51)	15	3.0C	9.2B	14.8B
	20	7.1B	15.2A	17.1A
	25	11.8A	13.4A	16.4AB
	30	11.4A	14.0A	15.9AB
	LSD	1.74	2.05	1.69
P. cryptogea (FWDM4)	15	4.6D	11.3C	15.3B
	20	8.8C	15.4A	17.4A
	25	14.1A	16.3A	17.8A
	30	12.5B	13.8B	14.7B
	LSD	1.32	1.08	1.32
P. citricola (PHP R-2)	15	6.4B	14.8B	19.4A
	20	9.6A	17.4A	20.4A
	25	9.2A	14.7B	16.4B
	30	6.3B	10.7C	12.4C
	LSD	0.85	1.62	1.56

[a]Data are means of two experiments each replicated two times
[b]Means in the same column for each isolate followed by the same letter are not significantly different according to Fisher's (protected) least significant difference test

Table 3.2 Effect of temperature on the growth rate (mean change in colony diameter per day) of five *Phyophthora* isolates in V8 broth after 72 h

	Growth rate (mm/day)[a]				
	P. citrophthora (GLN 7-23)	*P. parasitica (GLN 9-3)*	*P. citricola) (PHP R-2)*	*P. crytogea (FDM51)*	*P. cryptogea (FWDM4)*
Temp (C)					
15	5.6B[b]	1.5C	6.5A	5.8A	5.1B
20	6.7A	3.4B	6.8A	5.7B	5.8A
25	5.4B	5.2A	5.5C	5.4C	5.9A
30	5.8B	4.9A	5.8B	5.3C	4.9B
LSD	0.72	0.41	0.41	0.45	0.52

[a]Data are means of two experiments each replicated two times
[b]Means in the same columns followed by the same letter are not significantly different according to Fisher's (protected) least significant difference test

Table 3.3 Effect of temperature on mean number of sporangia in four microscope fields (X100) for five *Phytophthora* isolates

	Mean number of sporangia[a]				
	P. citrophthora (GLN7-23)	*P. parasitica* (GLN 9-3)	*P. citricola* (PHP R-2)	*P. crytogea* (FDM51)	*P. cryptogea* (FWDM4)
Temp (C)					
15	53.7B[b]	12.6B	40.2B	14.5C	33.0C
20	76.4B	17.3B	51.4B	41.9B	36.7C
25	149.6A	25.5A	116.1A	72.0A	96.5B
30	136.6A	0.2C	0.0	82.7A	131.2A
LSD	43.59	5.94	46.20	14.68	31.22

[a]Data are means of three experiments each replicated twice.
[b]Means in the same column for each isolate followed by the same letter are not significantly different according to Fisher's (protected) least significant difference test

Table 3.4 Effect of water temperature on mean percent germination of sporangial derived cysts of five *Phytophthora* isolates by zoospores or germ tubes

Isolate	Temp (C)	Mean % Germination[a] Zoospores	Germ tubes
P. cryptogea (FWDM4)	15	23.8C[b]	6.6A
	20	45.7A	5.6AB
	25	52.5A	4.5AB
	30	36.1B	3.5B
	LSD	9.45	2.58
P. cryptogea (FDM51)	15	11.4C	4.1C
	20	21.7B	4.9BC
	25	21.7B	7.1B
	30	39.2A	12.4A
	LSD	7.35	2.44
P. parasitica (GLN 9-3)	15	5.9AB	24.3B
	20	10.4A	46.4AB
	25	5.5AB	68.0A
	30	3.4B	59.0A
	LSD	4.59	22.72
P. citrophthora (GLN 7-23)	15	11.7AB	57.9A
	20	16.7A	60.1A
	25	15.1A	58.6A
	30	5.0B	67.4A
	LSD	7.41	NS
P.citricola (PHP R - 2)	15	10.3A	8.2A
	20	17.7A	6.2A
	25	14.6A	5.6A
	30	9.2A	4.2A
	LSD	NS	NS

[a]Data are means of three experiments each replicated four times.
[b]Means in the same column for each isolate followed by the same letter are not significantly different according to Fisher's (protected) least significant difference test

Table 3.5 Effect of water temperature on mean percent germination of cyst derived zoospore cysts of five *Phytophthora* isolates by zoospores or germ tubes

Isolate	Temp (C)	Mean %Germination[a] Zoospores	Germ tubes
P. cryptogea (FWDM4)	15	49.6AB[b]	6.3A
	20	50.3A	7.3A
	25	60.6A	6.0A
	30	39.0BC	4.3A
	LSD	11.32	NS
P. cryptogea (FDM51)	15	19.0B	6.0A
	20	22.6B	7.3A
	25	20.8B	8.6A
	30	40.8A	10.6A
	LSD	9.90	NS
P. parasitica (GLN 9-3)	15	34.2B	20.8B
	20	61.3A	13.0B
	25	43.0AB	49.7A
	30	24.5B	44.0A
	LSD	16.03	21.32
P.citrophthora (GLN 7-23)	15	22.6B	47.5B
	20	39.3A	44.3B
	25	33.5A	42.8B
	30	10.5C	69.0A
	LSD	7.21	12.22
P.citricola (PHP R - 2)	15	19.5B	12.8A
	20	47.0A	6.3A
	25	24.8B	6.1A
	30	21.3B	5.1A
	LSD	10.83	NS

[a]Data are means of three experiments each replicated four times
[b]Means in the same column for each isolate followed by the same letter are not significantly different according to Fisher's (protected) least significance difference test

CHAPTER IV

AUTONOMOUS DISPERSAL, ROOT LOCATION AND INFECTION BY SPORANGIAL AND CYST DERIVED ZOOSPORES OF *PHYTOPHTHORA* SPP. IN WATER

The most important role of zoospores in the life cycle of *Phytophthora* is serving as agents of dispersal and location of suitable infection courts (Donaldson and Deacon, 1993**;** Gow, 2004). Zoospores can disperse successfully locate, and colonize new sites by passive transport in water currents over the soil surface or through soil pores (Newhook *et al*., 1981). They have also been reported to disperse over short distances by active (autonomous) movement in slow moving or stationary soil water found between soil particles (Duniway, 1976; Newhook *et al*., 1981; Café-Filho *et al.,* 1992b). Passive dispersal is accomplished by moving irrigation water, streams, rivers or in rainfall runoff (Erwin *et al*., 1983; Café-Filho *et al.,* 1992b). Zoospore dispersal and direction of movement in these aquatic systems is mainly dependent on the dictates of water currents. Neher and Duniway (1992) found considerable dispersal of *Phytophthora parasitica* zoospores along the direction of water flow in irrigation furrows which was manifested by an increase in disease incidence with increasing distance from water inlet. This was interpreted to be a result of increased number of propagules transported away from the water inlet to areas of slower current where they settle out (Neher and Duniway, 1992). Café-Filho *et*

al. (1995) recorded long distance dispersal of viable propagules of *Phytophthora capsici* and *P. parasitica* with furrow irrigation practices downstream. Their study also found increased accumulation of secondary inoculum with distance and time from the initial inoculum source.

The ability of *Phytophthora* zoospores to swim and to locate suitable infection courts is a unique feature that offers great ecological advantage to these pathogens. Autonomous dispersal of *Phytophthora* zoospores over short distances or directed by chemotactic responses to host plant roots have been demonstrated (Hickman and Ho, 1966; Hickman, 1970). Motile zoospores have been observed to move autonomously and to accumulate at the zone of differentiation of elongating plant roots in soil (Ho, 1969; Kleijunas and Ko, 1976). Autonomous movement by zoospores in a particulate system composed of beads, sand, or mixtures of sand and soil has also been reported (Duniway, 1976; Newhook *et al*., 1981). More specifically, swimming distances of 12 mm have been reported for *P. cinnamomi* zoospores through capillary tubes (Allen and Newhook, 1973), 13 – 25 mm for *P. infestans* and *P. cinnamomi* zoospores in soil (Lacey, 1967), and 25 – 35 mm for *P. cryptogea* in the surface water of flooded soils (Duniway, 1976). These studies assessed autonomous dispersal in particulate systems where zoospores were exposed to continual collision with solid surfaces leading to ceasation of motility and premature encystment due to "contact stimulus". Because of inherent constraints to dispersal in these systems it has generally been concluded that zoospore autonomous dispersal is relatively insignificant and confers minimal or no ecological advantage to *Phytophthora*

spp. This line of thought has led investigators to direct their focus on passive dispersal of zoospores by moving water (Duniway, 1976; Newhook *et al*., 1981; Neher and Duniway, 1992; Café-Filho *et al.* 1992a, 1992b) with little attention to autonomous dispersal. However, location by zoospores of suitable infection courts on roots depends on additional lateral transport through wet soils or water, which must be provided for by their own autonomous dispersal (Café-Filho *et al.* 1992a). Currently, there is a noticeable lack of information in the literature on the role zoospore swimming plays in dispersal, location, and infection of plant roots by *Phytophthora* spp. in non-restraining liquid environments. Therefore, the ability of sporangial derived zoospore (SDZs) to move autonomously and to locate and infect seedlings was examined for some water borne *Phytophthora* spp.

Phytophthora spp. have the ability to produce different generations of zoospores in water. In the absence of a host or suitable substrate, the SDZs encyst and the resultant cysts can germinate by releasing further zoospores, or cyst derived zoospores (CDZs) (von Broembsen and Deacon, 1997; Xu and Morris, 1998; von Broembsen and Charlton, 2000). The CDZs are structurally similar to the SDZs (Xu and Morris, 1998; von Broembsen and Charlton, 2000). However, since zoospores depend entirely on endogenous energy reserves (Cerenius and Söderäll, 1984; Pennington *et al*., 1989), which cannot be renewed, the CDZs are likely to be depleted of energy having spent some of their macromolecules in cyst wall development and formation of new flagella. This may mean that the CDZs are deprived of internal sources of energy for their

swimming and location of new hosts. However, little is known regarding any component of biological fitness (including the ability to move autonomously and to locate and infect roots) of *Phytophthora* CDZs. Comparative studies of biological fitness of CDZs and SDZs may provide additional information for formulating better disease management strategies in irrigated agriculture.

MATERIALS AND METHODS

Culture and zoospore production. Zoospores were produced axenically from pure cultures of *Phytophthora parasitica* (Brenda de Haan) Dastur isolate GLN 9-3, and *P. cryptogea* (Perthybridge & Lafferty) isolates FDM51 and FWDM4 as previously described in Chapter III.

Maze experiments. The extent of lateral, active (autonomous) zoospore dispersal and root infection was assessed in model plastic mazes (19 x 11 x 5.5 cm) (Rubbermaid Incorp., Wooster, Ohio) each filled with 120 ml of sterile distilled water (SDW) to a depth of 14 mm. The mazes were constructed by partitioning plastic storage containers with 3" x 1" microscope slides (Fisher Scientific Co., Pittsburg, PA) glued with 100% Aquarium Sealant (Dow Corning Corp., Midland, MI) at distances of 15, 85, 150, and 210 mm from either side of a central inoculation point (Figs. 4.1, 4.2, and 4.2). The partitioning was meant, among other things, to limit the effect of water flow on the lateral movement of zoospores, which was to be used as a measure of active zoospore movement. It

was also meant to assess the ability of zoospores to swim around the ends of the glass slide partitions and cause infections at varying distances. Two14-d-old *Catharanthus roseus* seedlings were positioned at 15, 85, 150, or 210 mm from either side of the inoculation point (Figs. 4.1 and 4.2) in each maze. Mazes without plants were used as controls in each case.

Assessment of autonomous dispersal by SDZs in mazes. SDZs were harvested from plates as described previously and pipetted into a 100 ml, sterile Pyrex bottle. A portion of the zoospore suspension (about 60 ml in McCartney bottles) was set aside for use in production of CDZ as described below. An aliquot (1 ml of SDZ suspension) was pipetted into an Eppendorf tube, encysted by vortexing for 70 sec, and then used to determine zoospore concentration with a Spotlite haemacytometer (Baxter Healthcare Corp., McGraw Park, IL). The final concentration was adjusted to $10^3 – 10^4$ per ml. Two milliliters of the zoospore suspension were slowly inoculated into each maze by placing the pipette tip just under the water surface to minimize disturbance at the point of inoculation. After 4 h, the seedlings or 1 ml of liquid (from equivalent distances in mazes without plants) were plated on *Phytophthora* selective medium (P_{10}VPH) (Tsao and Ocaña, 1969; Masago *et al*., 1977). The selective medium (P_{10}VPH) consisted of 1 liter of CMA amended with 20 ml of P_{10}VPH stock solution (the stock solution was made up of vancomycin (1.0 g), 95% primaricin (0.05 g), 95% PCNB (0.53 g) and 99% hymexazole (0.25 g) in 100ml of sterile distilled water). The plates were incubated at 25 C for 48 h, after which the number of infections (# of hits)

per seedling pair and colony forming units (CFUs) were recorded for each distance. Isolates were tested in separate experiments on successive days. Each experiment consisted of six seedling pairs or water aliquots at each distance and the experiments were repeated three times.

Assessment of autonomous dispersal by CDZs in mazes. SDZs that had been set aside earlier were encysted by holding each McCartney bottle against a Maxi Mix II vortex machine for 2 min. Cyst suspensions from all the McCartney bottles were immediately mixed together in a sterile 100 ml Pyrex bottle and incubated for 7 - 8 h at 20 C (for *P. parasitica* GLN 9-3) or 25 C (for *P. cryptogea* isolates FDM51 and FWDM4). CDZ suspensions were harvested by pipetting out the upper third of liquid (~20 ml) from the Pyrex bottle and used without adjusting the concentration. Two ml of CDZ suspension were inoculated into each maze as outlined above. Seedlings or 1 ml of liquid (from control mazes) were removed at the test distances after 4 h and plated onto P_{10}VPH selective medium. The plates were incubated at 25 C for 48 h, after which the number of infections (# of hits) per seedling pair or colony forming units (CFUs) for mazes without seedlings were recorded for each distance. Isolates were tested in separate experiments on successive days. Each experiment consisted of six seedling pairs or water aliquots at each distance and the experiments were repeated three times.

Linear dispersal experiments. The ability of different generations of *Phytophthora* zoospores to move actively and infect roots in water were

assessed in linear V-shaped troughs (65 cm long x 2.9 cm wide at the top x 2.5 cm depth at the center) (Cameo Moulding Products, Nashville, TN) filled with 120 ml of SDW to a depth of 11 mm at center (Figs. 4.4 and 4.5). The linear troughs were constructed from commercially available gutter troughs and were sealed at either end to prevent water spill over. The troughs were set to level using a builder's level. The troughs were used to assess the ability of zoospores to disperse in linear paths devoid of any barriers and hence no frequent turns as in model mazes described previously. Two 14-d-old seedlings (*Catharanthus roseus* L.) were positioned at 0, 80, 160, 240, or 320 mm from either side of the central inoculation point (Figs. 4.3, 4.4, and 4.5). The seedlings were held in an upright position with their roots and root crown immersed in water using a layer of parafilm wrapped around the trough at the designated points (Fig. 4. 6). Linear troughs without seedlings were used as the control in each experiment.

Assessment of autonomous dispersal by SDZs in linear troughs. SDZs were obtained from each *Phytophthora* isolate as outlined previously and a portion (approximately 60 ml in McCartney bottles) was set aside for use in production of CDZs as described above. The concentrations of SDZs were adjusted to 10^3 – 10^4 and inoculated into each trough by pipette at point of inoculation as with mazes. Seedlings or 1 ml of liquid from control troughs were recovered from test distances after 4 h and were plated on *Phytophthora* selective medium (P_{10}VPH) as described previously. The plates were incubated at 25 C for 48 h, after which the number of infections (# of hits) per seedling pair or colony forming units

(CFUs) for control troughs were recorded for each distance. Isolates were tested in separate experiments on successive days. Each experiment consisted of six seedling pairs or water aliquots at each distance and the experiments were repeated three times.

Assessment of autonomous dispersal CDZs in linear troughs. CDZs were produced as described for maze experiments. Two ml of CDZs were used without adjusting concentration and inoculated into each trough as outlined previously. Seedlings or 1 ml of liquid (from control troughs) was obtained after 4 h and plated onto a selective medium (P_{10}VPH) and the plates incubated at 25 C for 48 h. The results were recorded as explained above. Isolates were tested on separate experiments on successive days. Each experiment consisted of six seedling pairs or water aliquots at each distance and the experiments were repeated three times.

Quantitative assessment of autonomous dispersal of SDZs and CDZs in linear troughs. Because the initial interest was to determine the maximum distance SDZs and CDZs could move autonomously in a non-restraining medium, CDZ concentrations were not adjusted accordingly in the studies described above. To achieve a quantitative comparison of the ability of SDZs and CDZs to disperse autonomously, the experiments described below were conducted. SDZs and CDZs were produced axenically from pure cultures of *Phytophthora cryptogea* (Perthybridge & Lafferty) isolates FDM51 and FWDM4

as described previously except that the concentration of both SDZs and CDZs were adjusted to 10^4 zoospores per ml before 2 ml of the suspension were added at the inoculation point of each trough. The results were recorded as explained previously. Isolates were tested on separate experiments on successive days. Each experiment consisted of four seedling pairs or water aliquots at each distance and the experiments were repeated three times.

Data analysis. Data from model maze and linear trough experiments were statistically analyzed using the PROC GLM (SAS Institute Inc., Cary, NC, 2001) for zoospore type infections (# of hits) and colony forming units (CFUs) at each distance.

RESULTS

Autonomous dispersal by SDZs and CDZs in mazes. SDZs and CDZs were able to actively move and infect 14-d-old *Catharanthus roseus* seedlings in 4 h in model mazes up to a distance of 215 mm except for SDZs of *P. parasitica* (GLN 9-3) and *P. cryptogea* (FWDM4) (Table 4.1), and CDZs of *P. cryptogea* (FDM51), which were capable of infecting seedlings up to a distance of 150 mm. SDZs and CDZs of all three isolates were recovered at all distances up to and including 215 mm (Table 4.2). For each *Phytophthora* isolate considered in this study, there was a trend for infection and recoverable colony forming units (CFUs) to decrease with increasing distance from the point of inoculation.

Autonomous dispersal by SDZs and CDZs in linear troughs. SDZs moved actively in linear troughs and infected 14-d-old *Catharanthus roseus* seedlings to a distance of 320 mm for all isolates (Table 4.3). Only CDZs of *P. parasitica* (GLN 9-3) caused seedling infections at a distance of 320 mm. SDZs and CDZs also were recovered at 320 mm in control linear troughs without seedlings (Table 4.4), except for *P. parasitica* (GLN 9-3). For each *Phytophthora* isolate considered in this study, there was a trend for infection rates and recoverable colony forming units (CFUs) to decrease with increasing distance from the point of inoculation.

Quantitative comparison of autonomous dispersal by SDZs and CDZs in linear troughs. When the same concentrations of *P. cryptogea* (FDM51) SDZs and CDZs were used, both zoospore types moved actively in linear troughs and infected 14-d-old *Catharanthus roseus* seedlings to a distance of 320 mm (Table 4.5). SDZs and CDZs of *P. cryptogea* (FWDM4) infected seedlings at distances of 240 and 320 mm, respectively (Table 4.6). Both SDZs and CDZs of *P. cryptogea* (FDM51) were recovered at 320 mm in control linear troughs without seedlings (Table 4.7). SDZs and CDZs of *P. cryptogea* (FWDM4) reached 320 and 240 mm, respectively (Table 4.8)

DISCUSSION

Phytophthora zoospores are important infective and dispersive units (Donaldson and Deacon, 1993; Gow, 2004). They are a stage in the life cycle not having cell walls or the ability to utilize exogenous energy sources, so zoospores must rely on their endogenous energy reserves for autonomous dispersal and maintenance of osmotic balance with their immediate environments (Suberkropp and Cantino, 1973; Pennington *et al*., 1989; Cerenius and Söderäll, 1984). The findings in this study that SDZs are able to autonomously disperse and infect roots in model mazes up to a distance of 215 mm and in linear troughs to a distance of 320 mm is unprecedented for *Phytophthora* spp. Ho and Hickman (1967) estimated that *Phytophthora megasperma* var. *sojae* zoospores could swim 560 mm in 1 h, if they swam in a fixed direction and at a constant speed. In practice, this movement is not achievable because according to Carlile (1986) and Deacon and Donaldson (1993), zoospore swimming is always interspersed with frequent spontaneous direction changes (Carlile, 1986; Deacon and Donaldson, 1993). On the basis of this assumption, it has been argued that autonomous zoospore dispersal is a means of thoroughly exploring a limited volume rather than traveling long distances (Carlile, 1986). Suh and Axtell (1999) were the first to demonstrate the ability of *Lagenidium giganteum* zoospores to travel and cause infection up to a distance of 600 mm. There is no report in the literature on *Phytophthora* zoospore movement to a comparable magnitude. The utilization of endogenous energy reserves by zoospores to fuel movement has been reported for *Blastocladiella emersonii* (Suberkropp and Cantino, 1973),

Phytophthora drechsleri (Barash *et a*l., 1965) and *P. palmivora* (Bimpong, 1975). Whatever the zoospore source, it is probable that zoospores depend on endogenous energy reserves to accomplish this feat.

The comparable infectivity by *Phytophthora* CDZs and SDZs of flood inoculated *Catharanthus roseus* seedlings has been reported (von Broembsen and Charlton, 2000). These studies also extend this observation to include the ability of CDZs and SDZs to move autonomously in a non-restraining medium and infect *Catharanthus roseus* seedlings up to a distance of 215 mm (in model mazes) and 320 mm (in linear troughs) and postulate that CDZs of *Phytophthora* spp. can serve as effective dispersal and infection units in irrigation water. The ability of zoospores to autonomously disperse up to these distances was apparently not influenced much by the gradient of root exudate components (Zentmeyer, 1961; Ho and Hickman, 1967; Hickman, 1970; Deacon, 1988), since they were also able to accomplish the same in control mazes and linear troughs seedlings. The current data somewhat parallel those of Crump and Branton (1966) who observed rapid and graceful swimming for secondary zoospores of *Saprolegnia* spp., which were able to travel greater distances than the primary zoospores.

The source of energy for *Phytophthora* CDZs remains largely unknown. The probable explanation for the observed ability of CDZs to perform as their SDZ counterparts may be partly because they were vortex encysted relatively soon (about 90 min) after release from the sporangia and so they did not have time to expend significant amount of endogenous energy. The method of vortex

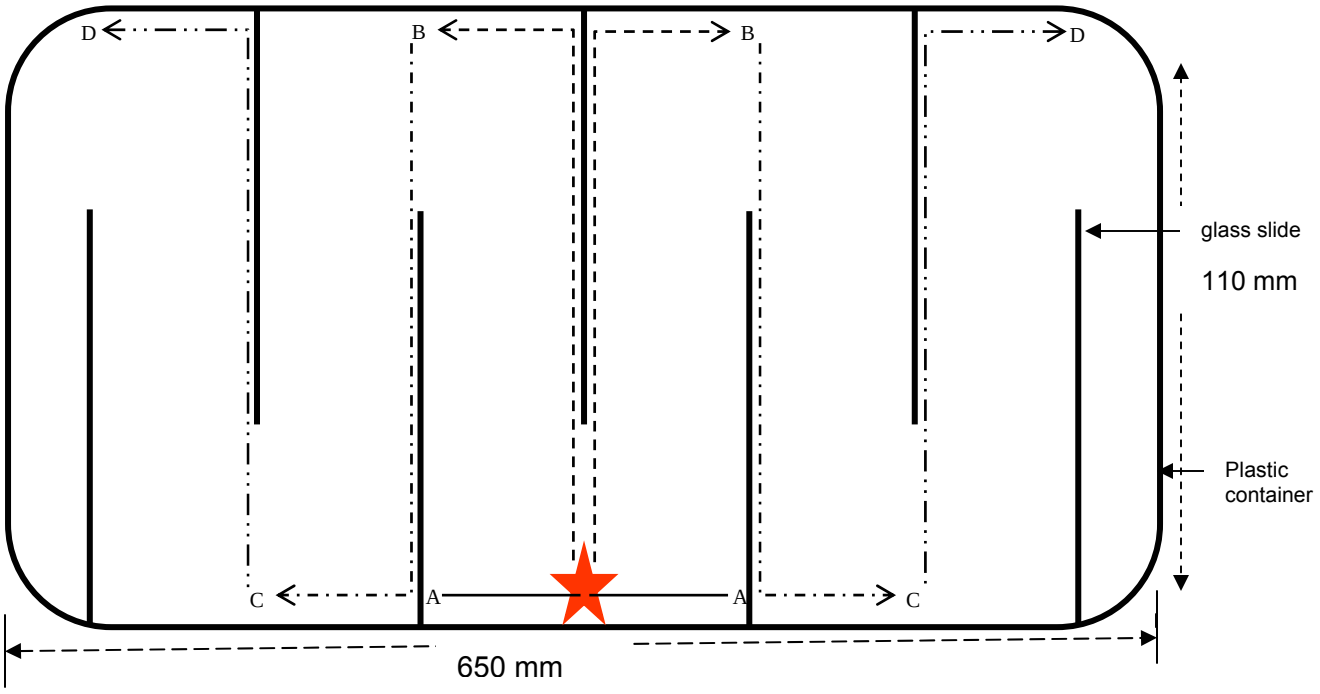
encystment soon after release was an attempt to mimic what could be happening to zoospores in irrigation water. According to Diéguez-Uribeondo *et al.* (1994) increasing zoospore swimming time may exhaust them of the storage materials needed by further generations. Increasing swimming time for SDZs before induction of encystment could be used to test this hypothesis.

SUMMARY AND CONCLUSIONS

1. *Phytophthora* zoospores are able move autonomously up to distances of 215 mm (in model mazes) and 320 mm (in linear troughs) with or without seedlings in water. Our results indicate that *Phytophthora* zoospores are able to move autonomously to greater distances in a non-restraining medium than previously thought.
2. The CDZs of *Phytophthora* spp. are able to move the same distances and cause infections. They can therefore play a significant role as dispersal and infection propagules of water-borne *Phytophthora* spp.
3. The results of the current study suggest that CDZs could have ecological roles to play and their presence should be factored into the available disease management protocols.
4. On the basis of our results and those of von Broembsen and Charlton (2000), we speculate that both SDZs and CDZs dispersal in non-restraining liquid systems like free surface water could be of greater

significance in the epidemiology of water-borne diseases in irrigated agriculture than previously considered.

Figure 4.1 Diagram of a model maze showing the inoculation point (red star), seedling positions, and distances from the inoculation point



Represents the inoculation point
A Represents the first seedling position, 15 mm from the inoculation point
B Represents the second seedling position, 85 mm from the inoculation point
C Represents the third seedling position, 150 mm from the inoculation point
D Represents the fourth seedling position, 215 mm from the inoculation point

Figure 4.2 A typical model maze with seedlings positioned at the longest distances equidistant from the central inoculation point

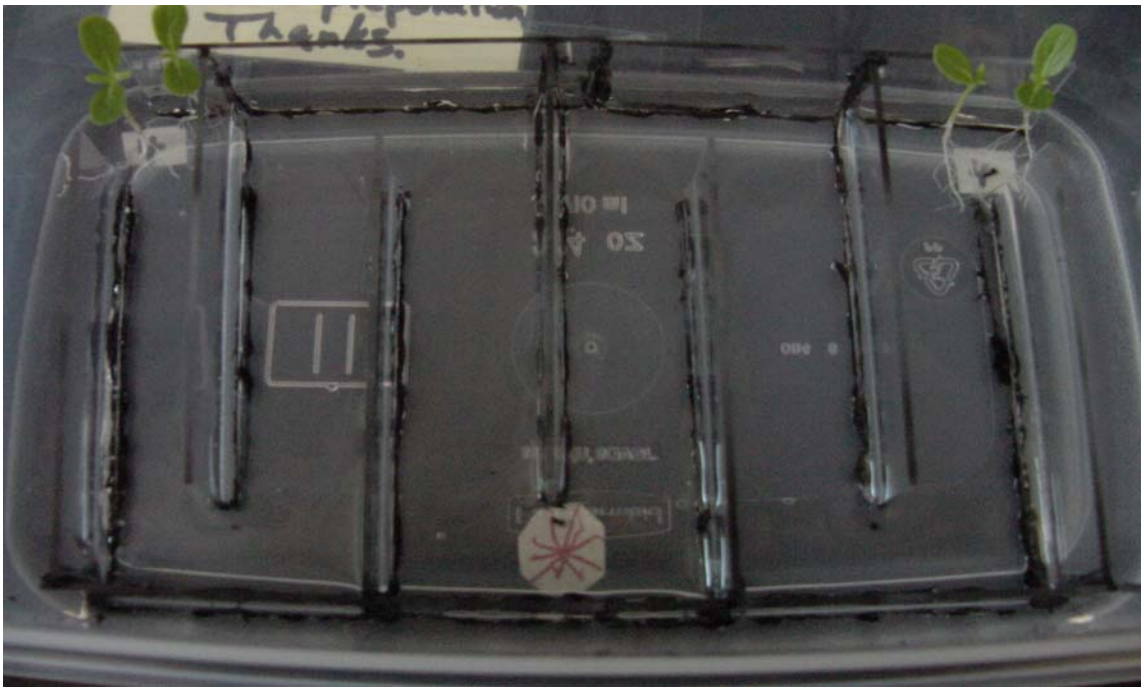
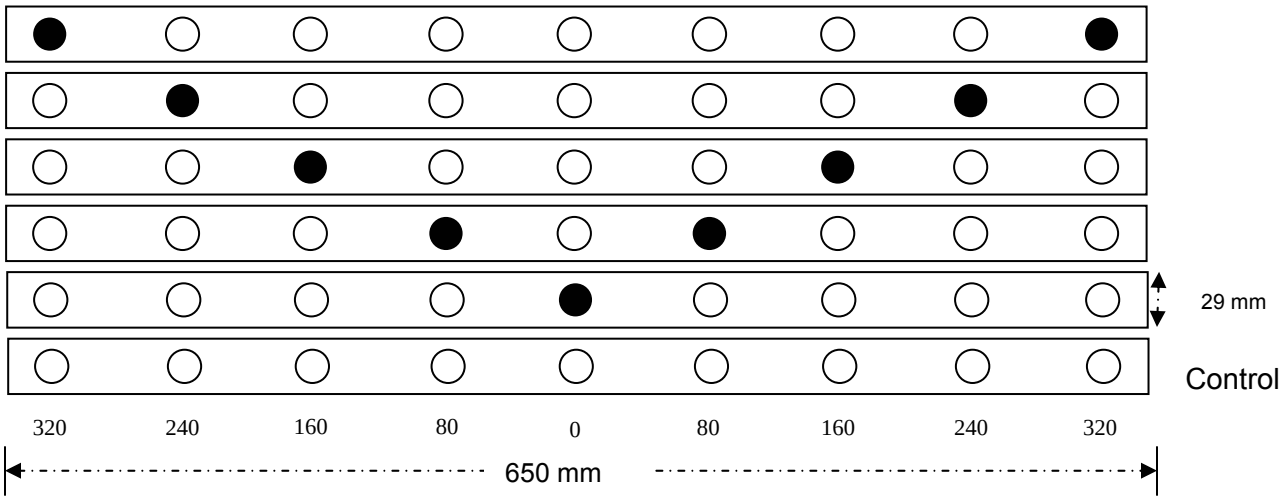


Figure 4.3 Maze experimental set-up showing seedling pairs at distances tested

Figure 4.4 Diagram of a block showing the point of inoculation (0 mm) in each trough and the positions of seedlings represented by black dots



0 mm is the inoculation point in each trough, control troughs were sampled at all distances (water samples)

● Represent position of seedlings relative to the inoculation point

○ Represent positions with no seedlings; water samples were taken from all positions in control troughs

Figure 4.5 A typical linear trough construction with seedling pairs in position

Figure 4.6 Linear troughs experimental set-up showing seedling pairs at positions equidistant from a central inoculation point

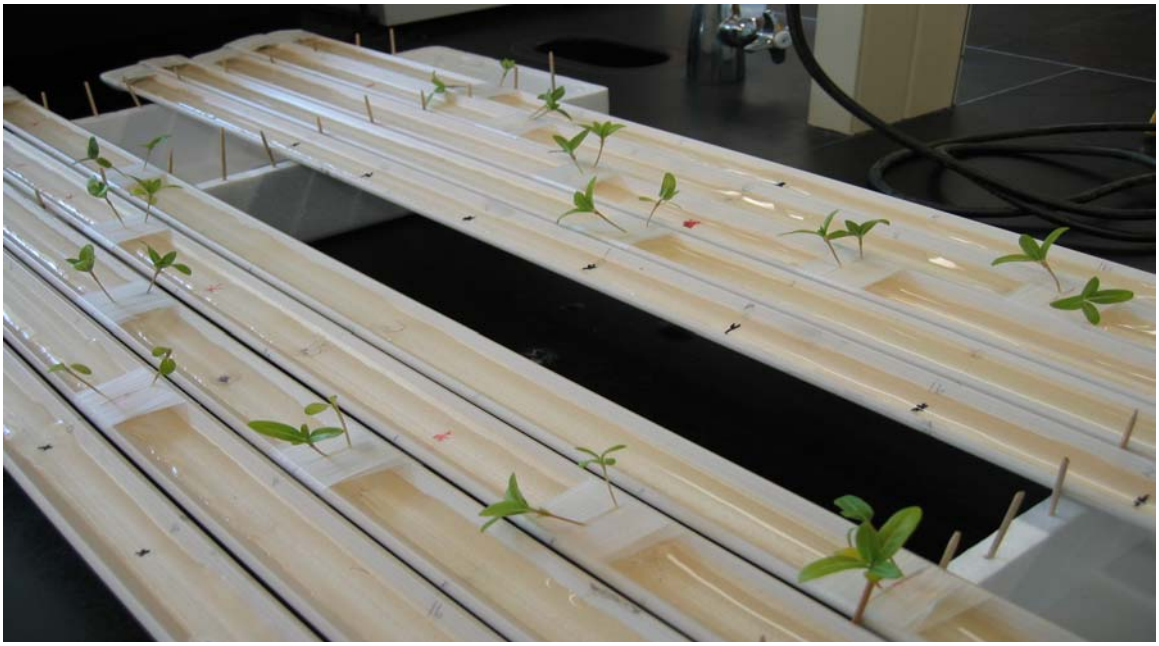


Table 4.1 Mean number of infections of *Catharanthus roseus* seedlings by sporangial derived zoospores (SDZs) and cyst derived zoospores (CDZs) of *Phytophthora* isolates at distances from inoculation points in model mazes after 4 h

Isolates	Distance (mm)	Replicates	Mean no. of infections[a]	
			SDZs	CDZs
P. parasitica (GLN 9-3)				
	15	9	6.0	4.2
	85	18	2.9	2.6
	150	18	0.8	0.3
	215	18	0.0	0.01
	LSD[b]		2.02	0.66
P. cryptogea (FDM51)				
	15	9	4.1	2.3
	85	18	3.3	2.0
	150	18	1.8	0.3
	215	18	0.1	0.0
	LSD		1.11	0.93
P. cryptogea (FWDM4)				
	15	9	6.0	3.7
	85	18	2.9	3.4
	150	18	0.8	0.8
	215	18	0.0	0.1
	LSD		2.02	2.04

[a]Data are mean number of infections of three experiments replicated six times
[b]Least significance difference of means at $P \leq 0.05$ within a single column for each isolate

Table 4.2 Mean number of colony forming units (CFUs) of sporangial derived zoospores (SDZs) and cyst derived zoospores (CDZs) of *Phytophthora* isolates recovered at distances from inoculation points in model mazes without seedlings after 4 h

Isolates	Distance (mm)	Replicates	Mean no. of CFUs[a]	
			SDZs	CDZs
P. cryptogea (FDM51)				
	15	9	48.5	32.5
	85	18	38.7	22.5
	150	18	16.0	1.1
	215	18	5.7	3.6
	LSD[b]		19.35	12.16
P. cryptogea (FWDM4)				
	15	9	85.9	60.6
	85	18	37.8	39.5
	150	18	6.0	8.9
	215	18	4.6	4.9
	LSD		32.77	28.5
P. parasitica (GLN 9-3)				
	15	9	49.5	57.8
	85	18	36.8	47.5
	150	18	4.8	8.2
	215	18	1.00	1.4
	LSD		12.02	41.52

[a]Data are means of number of colony forming units (CFUs) of three experiments replicated six times.
[b]Least significance difference of means at $P \leq 0.05$ within a single column for each isolate

Table 4.3 Mean number of infections of *Catharanthus roseus* seedlings by sporangial derived zoospores (SDZs) and cyst derived zoospores (CDZs) of *Phytophthora* isolates at distances from inoculation points in linear troughs after 4 h

Isolates	Distance (mm)	Replicates	Mean no. of infections [a]	
			SDZs	CDZs
P. cryptogea (FDM51)				
	0	9	9.0	5.5
	80	18	13.5	6.3
	160	18	5.2	4.2
	240	18	3.3	0.5
	320	18	1.2	0.0
	LSD[b]		4.21	3.81
P. cryptogea (FWDM4)				
	0	9	8.5	7.3
	80	18	10.5	6.7
	160	18	7.5	2.0
	240	18	0.5	0.2
	320	18	0.2	0.0
	LSD		3.3	3.3
P. parasitica (GLN 9-3)				
	0	9	8.0	6.1
	80	18	5.7	5.7
	160	18	3.2	0.3
	240	18	2.5	0.3
	320	18	0.8	0.2
	LSD		2.14	2.02

[a]Data are means of number of infections for three experiments replicated six times

[b]Least significance difference of means at $P \leq 0.05$ within a single column for each isolate

Table 4.4 Mean number of colony forming units (CFUs) of sporangial derived zoospores (SDZs) and cyst derived zoospores (CDZs) of *Phytophthora* isolates recovered at distances from inoculation points in linear troughs without seedlings after 4 h

Isolates	Distance (mm)	Replicates	Mean no. of CFUs [a]	
			SDZs	CDZs
P. cryptogea (FDM51)				
	0	9	73.0	53.5
	80	18	57.8	56.3
	160	18	31.0	27.3
	240	18	19.3	10.7
	320	18	7.7	2.7
	LSD[b]		45.78	24.06
P. cryptogea (FWDM4)				
	0	9	44.2	53.5
	80	18	40.5	56.3
	160	18	28.0	27.8
	240	18	5.5	10.7
	320	18	0.3	2.6
	LSD		19.6	17.42
P. parasitica (GLN 9-3)				
	0	9	251	80.8
	80	18	218	88.3
	160	18	83.5	21.6
	240	18	3.5	1.0
	320	18	1.7	0.0
	LSD		66.34	1.77

[a]Data are means of number of colony forming units (CFUs) of three experiments replicated six times

[b]Least significance difference of means at $P \leq 0.05$ within a single column for each isolate

Table 4.5 Mean number of infections of *Catharanthus roseus* seedlings by *Phytophthora cryptogea* (FDM51) sporangial derived zoospores (SDZs) and cyst derived zoospores (CDZs) at distances from inoculation points in linear troughs after 4 h

Isolates	Distance (mm)	Replicates	Mean no. of infections[a]
P. cryptogea (FDM51) SDZs			
	0	6	5.0A[b]
	80	12	3.0A
	160	12	0.6B
	240	12	0.7B
	320	12	0.2B
	LSD		2.05
P. cryptogea (FDM51) CDZs			
	0	6	3.5A
	80	12	1.7AB
	160	12	0.3B
	240	12	0.5B
	320	12	0.8B
	LSD		2.07

[a] Data are means of number of infections for three experiments replicated four times

[b] Means in the same column for each isolate followed by the same letter are not significantly different according to Fisher's (protected) least significance difference test

Table 4.6 Mean number of infections of *Catharanthus roseus* seedlings by *Phytophthora cryptogea* (FWDM4) sporangial derived zoospores (SDZs) and cyst derived zoospores (CDZs) at distances from inoculation points in linear troughs after 4 h

Isolates	Distance (mm)	Replicates	Mean no. of infections[a]
P. cryptogea (FWDM4) SDZs			
	0	6	4.2A[b]
	80	12	5.0A
	160	12	2.8B
	240	12	0.7C
	320	12	0.0C
	LSD		1.72
P. cryptogea (FWDM4) CDZs			
	0	6	5.0AB
	80	12	7.2A
	160	12	2.7B
	240	12	1.2B
	320	12	1.8B
	LSD		3.17

[a]Data are means of number of infections for three experiments replicated four times

[b]Means in the same column for each isolate followed by the same letter are not significantly different according to Fisher's (protected) least significance difference test

Table 4.7 Mean number of colony forming units (CFUs) of *Phytophthora cryptogea* (FDM51) sporangial derived zoospores (SDZs) and cyst derived zoospores (CDZs) recovered from linear troughs without seedlings at various distances at 4 h after inoculation

Isolates	Distance (mm)	Replicates	Mean CFUs[a]
*P. cryptogea (*FDM51*)* SDZs			
	0	12	48.9A[b]
	80	12	18.7B
	160	12	2.0C
	240	12	1.0C
	320	12	0.3C
	LSD		12.05
P. cryptogea (FDM51) CDZs			
	0	12	53.0A
	80	12	11.7B
	160	12	3.0B
	240	12	0.3B
	320	12	0.5B
	LSD		18.07

[a]Data are means of number of colony forming units (CFUs) of three experiments replicated four times

[b]Means in the same column for each isolate followed by the same letter are not significantly different according to Fisher's (protected) least significance difference test

Table 4.8 Mean number of colony forming units (CFUs) of *Phytophthora cryptogea* (FWDM4) sporangial derived zoospores (SDZs) and cyst derived zoospores (CDZs) recovered from linear troughs without seedlings at various distances at 4 h after inoculation

Isolates	Distance (mm)	Replicates	Mean CFUs[a]
P. cryptogea (FWDM4) SDZs			
	0	12	65.5A[b]
	80	12	56.5A
	160	12	44.5A
	240	12	35.7A
	320	12	31.0A
	LSD		NS
P. cryptogea (FWDM4) CDZs			
	0	12	54.7A
	80	12	39.5A
	160	12	22.8B
	240	12	3.3C
	320	12	0.0C
	LSD		15.46

[a]Data are means of number of colony forming units (CFUs) of three experiments replicated four times

[b]Means in the same column for each isolate followed by the same letter are not significantly different according to Fisher's (protected) least significance difference test

CHAPTER V

EFFECT OF TEMPERATURE ON MOTILITY RETENTION BY *PHYTOPHTHORA* SPORANGIAL AND CYST DERIVED ZOOSPORES

The flagellated zoospores of *Phytophthora* spp. utilize a motile phase upon release from sporangia, a unique attribute that enables them to respond to environmental cues thereby offering great ecological advantage (Deacon, 1996). Motility offers zoospores the advantage of being able to respond to environmental cues such as living plant roots (Kleijunas and Ko, 1976). Motile zoospores are able to migrate to and collect in favorable infection courts, thus increasing their inoculum potential. Migration and accumulation on plant roots in soil by motile zoospores have been reported for *P. megasperma* (Ho, 1960), *P. drechsleri* (Mehrotra, 1970) and *P. palmivora* (Kleijunas and Ko, 1976). The longer that zoospores swim, the greater are their chances of reaching infection sites (Sato, 1979) compared to those that encyst before reaching an infection court.

There is little information on factors that influence the motile zoospore stage but some observations have been made for temperature effects on zoospore motility (Ho and Hickman, 1967; Bimpong and Clerk, 1970; Sato, 1979; Carlile, 1986; Roy, 1999). Ho and Hickman (1967) reported on the influence of

relatively low temperatures to prolong zoospore motility in liquid systems. Carlile (1986) also reported that cool water (10 – 15 C) resulted in the longest swimming periods.

Phytophthora zoospores are capable of producing successive generations of zoospores in water (already discussed in Chapter III). The second generation zoospores that emerge from cysts during the repeated emergence are structurally similar to those released from the sporangia (Xu and Morris, 1998; von Broembsen and Charlton, 2000)**.** However, since zoospores depend entirely on endogenous energy reserves which cannot be renewed (Cerenius and Söderäll, 1984; Pennington *et al*., 1989), cyst derived zoospores (CDZs) may become depleted of energy by utilizing critical macromolecules in cyst wall development and formation of new flagella. This may mean that the CDZs are deprived of internal sources of energy for their swimming activities. A comparative study of the attributes of sporangial derived zoospores (SDZs) and cyst derived zoospores (CDZs) for any *Phytophthora* spp. is completely lacking in the literature. Two *Phytophthora cryptogea* isolates (isolates FDM51 and FWDM4) were selected based on their ability to produce CDZs over a wide temperature range (observed in Chapter III).

This chapter presents the results of a comparative study of ability of SDZs and CDZs of these two isolates to retain motility and of the role of temperature in retention motility.

MATERIALS AND METHODS

Assessment of SDZ motility retention. SDZs were produced axenically from *Phytophthora cryptogea* (isolates FDM51 and FWDM4) as previously described. Immediately after release, 3 ml of zoospore suspension were pipetted into 65 mm petri dishes and three replicate dishes were incubated at 15, 20, or 25 C. Zoospore motility retention was assessed at 0, 3, 6, and 9 h as follows: the numbers of motile zoospores at three randomly selected fields in each plate were focused near the top of liquid and examined at 200X with the aid of a video-micrograph as outlined below.

Assessment of CDZ motility retention. A batch of SDZs (in about 80 ml) was vortex encysted by holding a 100 ml Pyrex bottle against a Maxi Mix II vortex machine (Whatman Inc., Clifton, NJ) for 3 min. At 6 – 7 h post encystment CDZs were harvested by carefully pipetting out the upper third of liquid from the Pyrex bottle and into a different sterile Pyrex bottle. Three ml of this CDZ suspension were pipetted into each 65 mm petri dish to give three replicate dishes, which were incubated at 15, 20, or 25 C. Motility retention of CDZs was determined at 0, 3, 6, and 9 h using video-microscopy as described below.

Video-microscopy. The motility retention of both SDZ and CDZs were examined using video-microscopy as previously described (Jones *et al*., 1991). Briefly, a video camera (Sony DXC-151A) without front lenses was attached by a

photographic extension tube to a Nikon Labophot microscope (Nikon Corp., Chiyoda-Ku, Tokyo). The camera was attached through a Sony CMA-D2 camera adaptor power supply unit to a Sony SVO-1610 HQ video recorder. The video recorder was in turn attached to a Sony Triniton 15" monitor. Recordings were made using light transmitted from a Philips 6V 20W bulb at 200X. The camera used contained integral time-date facility, which superimposed times (as minutes/seconds/tenths of seconds) on the video recordings. The zoospores (both SDZs and CDZs) were tape-recorded over 3 – 7 sec in three randomly selected microscope fields for each plate. The status of zoospores (whether motile or non-motile) was assessed by first circling zoospores on clear plastic wrap laid over the monitor, advancing the tape for 3 sec, then scoring zoospores that have moved out of the circles as motile. Most zoospores moved off the screen, but a minimum distance scored as motile was movement of three zoospore lengths from the circle. Each experiment consisted of three replicate plates and each experiment was repeated three times.

Data analysis. Data obtained from all the isolates were statistically analyzed using the PROC GLM (SAS Institute Inc., Cary, NC, 2001) for zoospore type X temperature interactions.

RESULTS

Effect of temperatures on motility retention by SDZs. Motility at the start of these experiments was <100% (Fig. 5.1). For *P. cryptogea* (FWDM4) SDZs at 12

h after incubation, there was a reduction in mean percent motility ($P \leq 0.05$) to 78.7% at 20 C and to 46.9% at 25 C. For *P. cryptogea* (FDM51), after 9 h incubation at 15, 20, and 25 C mean percent motility was greatly reduced for all temperatures. Incubation at 25 C resulted in the greatest reduction in motility. Thus, compared with SDZs of *P. cryptogea* (FWDM4), those of *P. cryptogea* (FDM51) had shorter motile periods at all three temperatures.

Effect of temperature on motility retention by CDZs. Motility at the start of these experiments was <100% (Fig. 5.2). For CDZs of *P. cryptogea* (FWDM4), the mean percentage motility dropped significantly ($P \leq 0.05$) by 9 h for all temperatures tested. The mean percent motility of *P. cryptogea* (FDM51) CDZs was less than <40% immediately after harvest and no motile CDZs were present after 3 h incubation at 25 C.

DISCUSSION

These studies provide an extensive analysis of the effect of temperature on SDZs and also compare the effects of temperature on CDZs. To our knowledge, these are the first studies measuring zoospore motility at three temperatures over a period of 12 h for any *Phytophthora* isolate. The experimental data confirm and extend previous observations on the ability of low temperatures to prolong *Phytophthora* SDZ motility in liquid systems (Ho and Hickman, 1967; Sato, 1979; Carlile, 1986; Roy, 1999). Based on this evidence of the effect of temperature on zoospore motility retention, it appears that

moderately cool temperatures may be more favorable for spread of *Phytophthora* spp. in irrigated agriculture compared to higher temperatures.

CDZs had inferior motility retention compared with their SDZ counterparts, but low temperatures also supported better motility retention for these zoospore types. This may be because it is generally thought that CDZs do not have as high endogenous energy reserves as SDZs. This in part may account for their low motility retention at high temperatures. More accurate estimates of motility retention would be possible if factors that influence more transitions are clearly understood and considered.

SUMMARY AND CONCLUSIONS

1. The two isolates of *P. cryptogea* considered in this study had different capacities for motility retention.
2. Overall, the zoospores of both isolates (FDM51 and FWDM4) of *P. cryptogea* were able to retain motility for longer time at the lowest water temperature (15 C) than at the highest water temperature (25 C). This was especially evident for CDZs compared to SDZs.
3. For both isolates CDZs were not able to retain motility as well as SDZs.

Figure 5.1 Effect of temperature on mean percent motility of sporangial derived zoospores (SDZs) of *Phytophthora cryptogea* (isolates FWDM4 and FDM51) after 3, 6, 9, or 12 h incubation at 15, 20, or 25 C

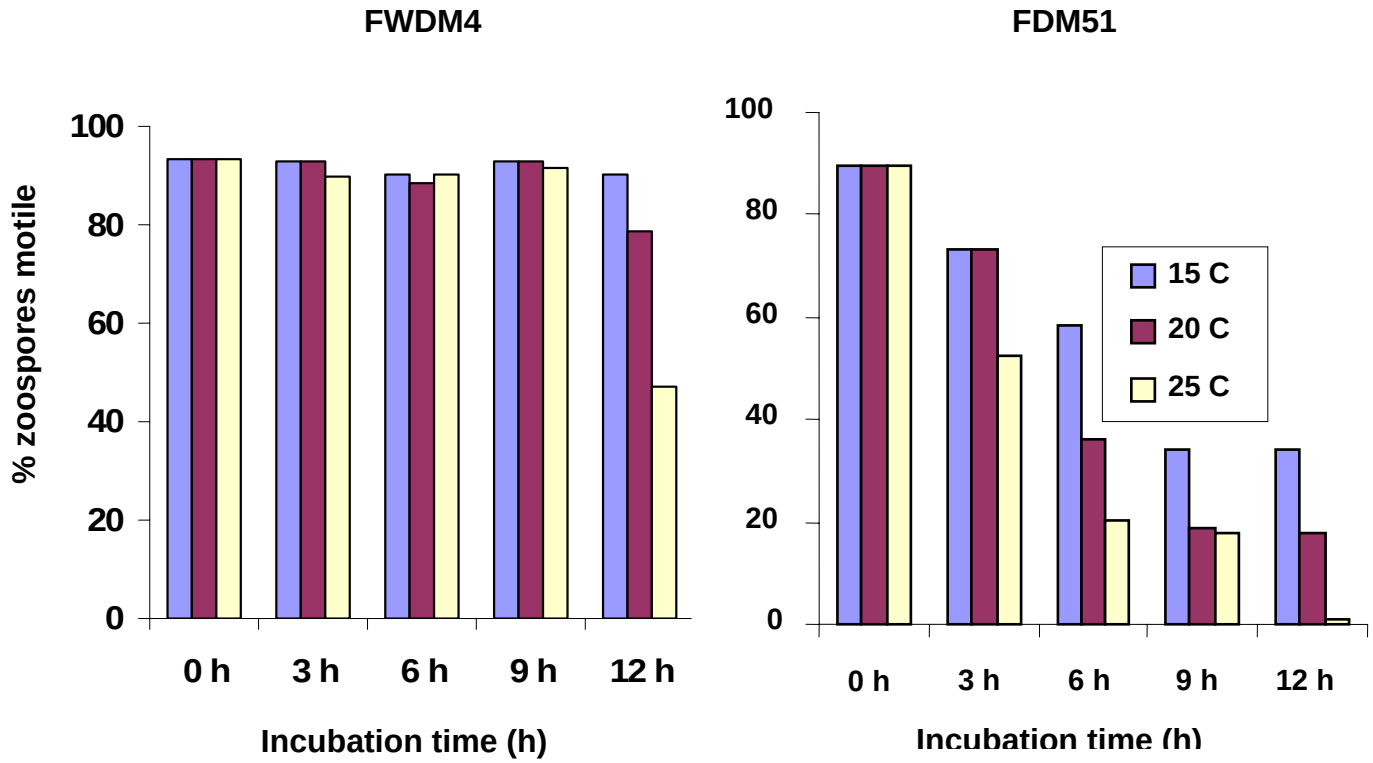
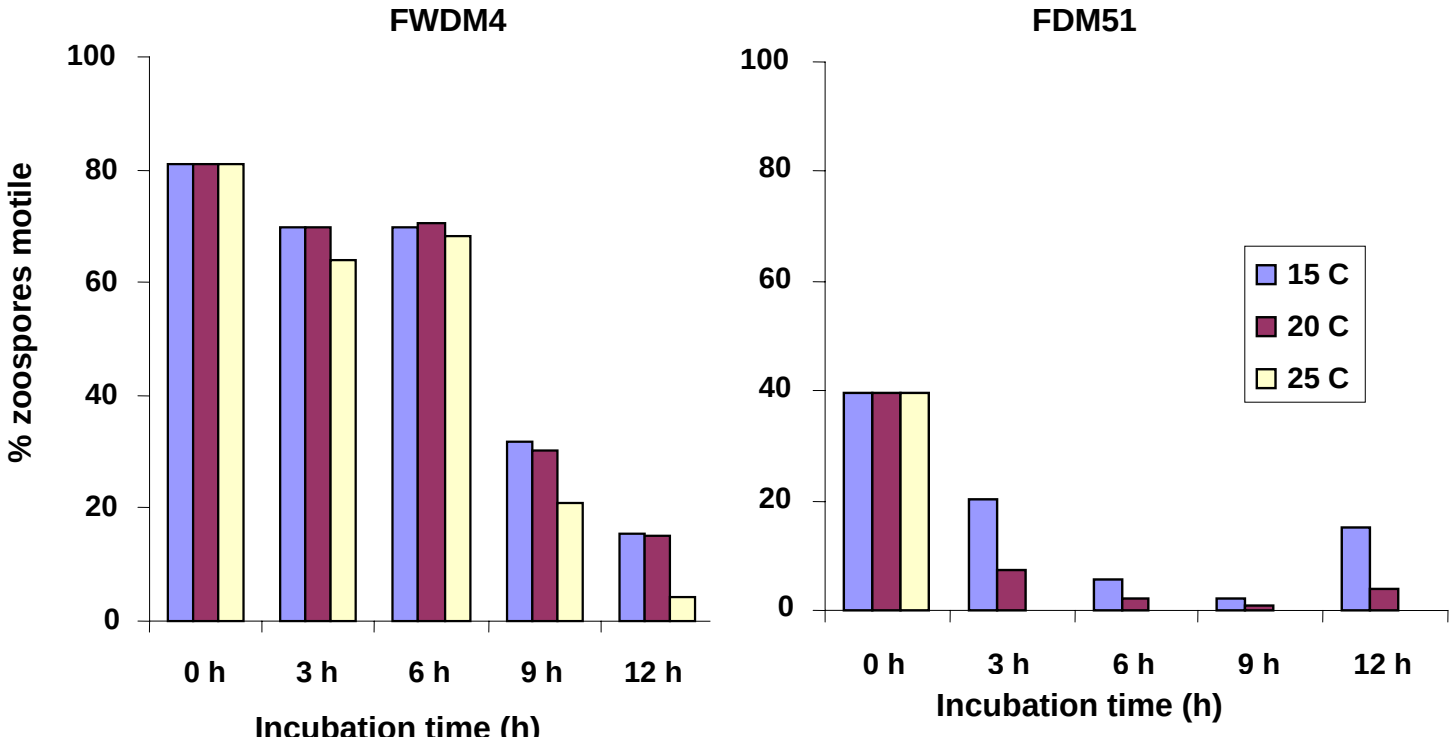


Figure 5.2 Effect of temperature on mean percent motility of cyst derived zoospores (CDZs) of *Phytophthora cryptogea* (isolates FWDM4 and FDM51) after 3, 6, 9, or 12 h incubation at 15, 20, or 25 C



CHAPTER VI

SIGNIFICANCE OF RESEARCH

The occurrence of phytopathogenic *Phytophthora* spp. in irrigation water was first reported by Bewley and Buddin (1921). Since then, there have been numerous reports of successful recovery of *Phytophthora* spp. from water bodies including nursery irrigation water (Klotz *et al*., 1959; McIntosh, 1966; Thomson and Allen, 1974; Kleijunas and Ko, 1976; Shokes and McCarter, 1979 and Ali-Shtaye *et al*., 1991; Pittis and Colhoun, 1984; von Broembsen, 1984, 1990; MacDonald *et al*., 1994; von Broembsen and Wilson, 1998; Yamak *et al*., 2002; Bush *et al*., 2003). Zoospores constitute the dominant asexual *Phytophthora* propagules that occur in irrigation water (Thomson, 1972; von Broembsen and Charlton, 2001) and are responsible for disease outbreaks when zoospore contaminated or recycled irrigation water is used on susceptible nursery crops. The current study on the biology of isolates of water-borne *Phytophthora* spp. has important implications for decision making support systems.

Effect of temperature on asexual propagules of water-borne *Phytophthora* spp. *Phytophthora* spp. are oomycetous plant pathogens with complex asexual

life cycle stages each of which constitute infectious propagules, notably the mycelium, sporangia, zoospores, and zoospore cysts (de Souza *et al*., 2003). Each stage of *Phytophthora* pathogenesis is influenced differently by environmental factors such as temperature. This in turn influences the geographic distribution and seasonality of *Phytophthora* diseases (Duniway, 1983). The information obtained in this study could be used to time disease control measures so that they coincide with periods when production of asexual propagules of *Phytophthora* spp., especially zoospores, are high.

***Phytophthora* repeated emergence.** The ability of *Phytophthora* zoospore cysts to germinate by releasing further zoospores in water (repeated emergence) is a significant finding with implications for survival and spread in irrigation water, especially in the absence of available hosts or suitable substrates (Cerenius and Söderhäll, 1985; Diéguez-Uribeondo *et al*., 1994; von Broembsen and Deacon, 1997**;** von Broembsen and Charlton, 2000). The intervening encystments probably function to provide zoospores the opportunity to replace worn out flagella and then to re-emerge to disperse and thoroughly explore their environment (Salvin, 1940)**.** The observed influence of water temperature on *Phytophthora* mode of zoospore cyst germination should be factored in when considering the epidemiology and management of these pathogens.

Motility retention by sporangial and cyst derived zoospores. Motile zoospores have the ability to migrate and collect in favorable infection courts,

thereby increasing their inoculum potential. The longer the duration of the zoospore motile phase, the higher the chances of causing infection would be. Cyst derived zoospores lost motility sooner than sporangial derived zoospores, especially at the highest water temperature (25 C), but this varied considerably with isolates. Both types of zoospores retained motility longer at the lowest water temperature (15 C), suggesting that cool temperatures would be more favorable for *Phytophthora* spread in irrigated agriculture.

Autonomous dispersal and root infection by sporangial and cyst derived zoospores. Both sporangial and cyst derived zoospores move to, locate, and infect roots at distances up to 215 mm (in model mazes) and 320 mm (in linear troughs) and were also able to do the same in the controls without seedlings. This demonstrated that zoospores have the ability to move further autonomously in non-restraining media than previously thought. This capability is a unique feature that probably offers these pathogens great ecological advantage. The additional generations of zoospores could be important survival structures that aid in dispersal especially in the absence of hosts or suitable substrates in irrigation water (Cerenius and Söderhäll, 1985; Diéguez-Uribeondo *et al*., 1994; von Broembsen and Deacon, 1997). Moreover, these experimental data and that of von Broembsen and Charlton (2000) point to the fact that cyst derived zoospores of *Phytophthora* spp. can potentially serve as infection and dispersal units in irrigation water. Reappraisal of *Phytophthora* disease management strategies in irrigated agriculture should consider this new information.

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