High Temperature Predisposition of Sweet Pepper to Pythium Root Rot and its Remediation by *Pseudomonas chlororaphis*

by

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Pythium root rot caused by *Pythium aphanidermatum*, a destructive disease of sweet pepper and other hydroponic crops, is characterized by root browning (necrosis) and reduces growth of roots and shoots. Serious losses in crop productivity are common, in part for lack of adequate control measures. Severe root rot has been previously associated with episodes of high temperature, but whether this is due to high temperature effects on the host, the pathogen, or their interaction remains unclear. To clarify these relationships, and to provide a basis for predicting rapid increases in root rot, quantitative experiments were conducted to determine how episodes of high root-zone temperature are associated with root browning. Pepper plants were grown separately in hydroponic units containing aerated nutrient solution positioned in temperature-controlled water baths. The root zone temperature was 23°C except
during high temperature treatments. Browning developed progressively earlier in roots that were maintained at 33°C for 9 to 144 h immediately before they were inoculated with zoospores of *P. aphanidermatum*, and in all instances earlier than in control plants maintained continuously at 23°C. The data demonstrated unequivocally that high root-zone temperature can predispose pepper plants to Pythium root rot. Browning also developed earlier when root inoculation with *P. aphanidermatum* was delayed as long as 216 h following exposure at 33°C for 72 h, indicating that predisposition of the host by high temperature episodes can persist for at least 9 days. The ability of *Pseudomonas chlororaphis* strain 63-28 to suppress Pythium root rot and promote plant growth was investigated in pepper plants grown in the hydroponic units and predisposed to the disease, i.e. plants were predisposed to high temperature (33°C for 72 h ending at 3 days before inoculation) or not predisposed (constant 23°C). When *P. chlororaphis* was applied in the nutrient solution at a final density of $10^7$ CFU mL$^{-1}$ 7 days before the high temperature episode, the agent delayed root browning, re-mediated predisposition to root rot, and increased growth of plants that were and were not inoculated with *P. aphanidermatum*. It is concluded that high temperature predisposed pepper seedlings to root rot and that strain 63-28 has substantial potential for managing root rot regardless of predisposition by high temperature.
Preface

This thesis contains two articles that have been published in the peer-reviewed scientific journal Tropical Plant Pathology. The co-author contributed intellectually in writing the papers. All experimental work was performed by Coralie Sopher.

CHAPTER 2


CHAPTER 3

Acknowledgements

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1. Introduction and literature review

1.1. Introduction

Pythium root rot (PRR) is ubiquitous and frequently destructive in sweet pepper and most other kinds of crops produced in hydroponic systems, including tomato, cucumber, lettuce, spinach, arugula, and cut flowers such as snapdragon, rose and chrysanthemum (Zheng et al., 2000; Sutton et al., 2006; Stewart-Wade, 2011). Epidemics of PRR continually threaten the productivity and profitability of hydroponic greenhouse vegetables and ornamental crops in Canada, the USA, European countries, Brazil and elsewhere (Bates and Stanghellini, 1984; Sutton et al., 2006; Corrêa et al. 2010). The disease has been particularly severe in crops grown in hydroponic systems in which the plant nutrient solution is circulated continuously in the root zone (Stanghellini et al., 1996; Sutton et al., 2006; Stewart-Wade, 2011) The main cause of PRR in hydroponic crops is *Pythium aphanidermatum* (Edson) Fitzpatrick (Sutton et al., 2006), but *P. dissotocum* Drechsler, *P. ultimum* Trow var. *ultimum* and members of *Pythium* group F have also been reported as causes for this disease (Jarvis, 1992; Moulin et al. 1994; Owen-Going et al., 2002; Sutton et al., 2006).

PRR develops in two principal phases, a biotrophic phase during which roots are colonized by *P. aphanidermatum* but remain symptomless, and a necrotrophic phase in which colonized roots turn brown and often fragment. Growth and productivity of sweet peppers and other plants are usually reduced only after the roots turn brown. Control of root browning is critical for maintaining high productivity of *P. aphanidermatum*-infected crops, but effective and practical
methods to achieve this have not been developed. A major difficulty is that the
disease typically becomes severe during or following periods of warm or sunny
weather when the root-zone temperature is high (e.g. 28-34°C). High
temperature favours the shift from the biotrophic phase to the necrotrophic phase
and rapid root rot development. Root zone temperatures that are sufficiently low
to maintain the biotrophic phase and thereby avoid the destructive necrotrophic
phase are sub-optimal for pepper plant growth and difficult and prohibitively
expensive to maintain. Beneficial microbes are a potential means for controlling
root rot and are normally safe for greenhouse employees, consumers, and the
environment, but have received only limited attention in sweet peppers. To be of
value against PRR in hydroponic sweet pepper crops in Ontario, it can be
expected that a microbe would have to suppress root rot satisfactorily during
periods of high as well as moderate temperatures. Evidence indicates that a
quantitative understanding of high temperature episodes in relation to root rot
and beneficial microbes may be fundamental for developing successful
management of PRR.

This literature review provides a broad background perspective of the
epidemiology of PRR and a rationale for the investigations of host predisposition
to the disease and biological control. The main components of Pythium root rot
epidemics are the crop environment (i.e. the hydroponic system), the host (sweet
pepper) and the pathogen (P. aphanidermatum), are considered first. Next, relevant
details are provided of the epidemiology of PRR in sweet peppers,
including inoculum sources, survival and dispersal of the pathogen and progress of the disease in pepper crops.

Temperature, a variable recognized as a principal factor affecting PRR epidemics is considered in detail in relation to growth of sweet peppers, reported effects on the progress of PRR, and possible predisposition of sweet peppers to the disease. Plant stress and host responses to abiotic (e.g. environment) and biotic stress (e.g. the pathogen) factors are reviewed. Next disease management practices including the use of beneficial bacteria to suppress PRR are presented. Background information on the beneficial microbe *Pseudomonas chlororaphis* and its use for managing PRR, followed by a rationale for work done in this thesis and the thesis objectives complete the literature review.

1.2. Hydroponic systems

Plants in hydroponic culture are grown without natural soil and obtain elements essential for growth and development from nutrient solution provided in the root zone. In regions with cool-temperate climates, such as Southern Canada, hydroponic crops normally are grown in large greenhouse complexes with substantial control of root-zone conditions and the aerial microclimate. Individual hydroponic systems typically accommodate tens of thousands of crop plants. In many systems, nutrient solution is stored or pre-mixed in large tanks and subsequently is pumped or flows under gravity into pipes, through the root zone of the crop, and back to the tanks (illustrated in Sutton et al., 2006). The organic and inorganic composition, pH and electrical conductivity (EC) of the
nutrient solution are monitored, restored or adjusted as the solution passes through the tanks. Present-day hydroponic systems normally are “closed” such that nutrient solution is repeatedly re-circulated through the root zone of the crop. Compared to “open” systems, in which used nutrient solution is discharged outside the greenhouse, closed systems help to conserve water and nutrients and minimize environmental contamination with plant nutrients (Menzies et al., 1996; Van Os et al., 1999). Water quality is of primary importance for growing hydroponic crops, especially in closed systems (Graham et al., 2011; Stewart-Wade, 2011). Sources used include wells, surface ponds, lakes, municipal supplies and springs (Hong and Moorman, 2005; Sutton et al., 2006). For disease management it is important that the water be free from Pythium and other pathogens (Sutton et al., 2006; Stewart-Wade, 2011). Several factors that contribute to this include the use of new or sanitized materials and disinfection of hydroponic systems. Chlorine levels should be sufficiently low to avoid formation of compounds in the nutrient solution that adversely affect plant health. In some instances, chlorine levels in some water sources can inactivate beneficial microbes. In contrast to outdoor crops grown in natural soils, hydroponic crops are largely protected from adverse weather conditions, such as fluctuating water availability and temperature extremes and can be grown year-round.

Substantial levels of control of root-zone conditions and aerial microclimate are normally achieved in greenhouse hydroponic systems in Canada. Sophisticated instrumentation is employed that automatically monitors and regulates (within limits) the nutrient composition, dissolved oxygen (DO)
concentration, pH, flow rate and temperature of the nutrient solution as well as light intensity, temperature, humidity and CO₂ levels in the crop canopy. Many hydroponic systems also include equipment to kill or inactivate plant pathogens as the solution circulates outside of the crop. Examples include those that treat the solution with UV light, ultrafiltration, heat, chemical disinfectants, surfactants and aqueous ozone (Stanghellini et al., 1996; Graham et al., 2011; Stewart-Wade, 2011). Competent management skills combined with substantial and broad practical knowledge of hydroponic systems are requisites for successful crop production in these hydroponic greenhouses.

Most hydroponic systems employed in Canada involve large capital and operating costs so are generally used for producing crops of high economic value. Taken together, the high cost inputs for hydroponic crops underscore the need to minimize crop losses due to PRR and other factors, especially given the narrow profit margins that commonly prevail and the vagaries of market and export conditions. The economics of hydroponic crop production also highlights the potential benefits of new products that promote the health, production efficiency and productivity of marketable yield. Beneficial microbes are in the forefront of emerging products capable of promoting health and productivity of hydroponic crops and in some instances have the potential to reduce costs of crop production such as by reducing the need for fertilizer (Bloemberg and Lugtenberg, 2001, Sutton et al., 2012).
1.3. Production of sweet pepper in hydroponic systems in Ontario

Hydroponic sweet peppers (*Capsicum annuum* L.) accounted for 32% (247 hectares) of the total area of greenhouse vegetable production in Ontario in 2011 (Harrison, 2011) compared to 42% for tomatoes and 27% for cucumbers. About 61 million kg of peppers are currently grown each year in Ontario greenhouses (93 million kg in Canada), representing approximately $270 million in sales (Statistics Canada, 2011).

Sweet peppers used for hydroponic production are usually indeterminate and genetically-uniform F-1 hybrids. Like all peppers, hybrid peppers are members of the Solanaceae family. Pepper crops are initially grown from seeds in greenhouses dedicated to transplant production. The seeds are generally planted in small rockwool plugs and transferred to rockwool cubes about 2 weeks later as the first true leaves emerge. At approximately 6 weeks after seeding, when about 25 cm tall, the plants are transplanted to growing slabs (e.g. rockwool, coir) production greenhouses with closed hydroponic systems. In some instances plants in rockwool cubes are positioned in troughs and the roots grow out into circulating nutrient solution. More commonly, however, the cubes are positioned on slabs of a rooting medium such as rockwool or coconut fibre (coir). Usually three plants are spaced along 90-cm long slabs arranged in rows to give a plant density of about 32,000 plants ha$^{-1}$ (Gillian Ferguson, OMAFRA, personal communication). The roots grow down through the slabs which provide physical support. Circulating nutrient solution enters the root zone near each plant via an irrigation emitter inserted into the rockwool cube, and subsequently drains from
the root zone into a collection trough and flows back to the mixing tank. For many years growers positioned the slabs of rooting medium on plastic sheeting on the greenhouse floor which was angled towards the drain. The recent trend however is to suspend the slabs above the greenhouse floor on suspended troughs that allow height adjustments as plants increase in height, increase airflow, improve nutrient solution flow, easier harvesting and a more sanitary environment. The raised troughs also help maintain cool irrigation solution temperatures since irrigation tubing is concealed below the benches away from direct sunlight.

Stems and foliage of hybrid peppers grow continuously for as long as 8-10 months or more and form dense canopies. Stems require frequent tying to vertical support strings. Fruit production begins at about ten weeks after transplanting but an additional three weeks is needed to achieve red, orange or yellow pigmentation in the fruits. Fruit production is normally allowed to continue for about 8 months. Sweet peppers require pollination and fertilization for fruit development and are self-pollinating. Although sweet peppers do not require a vector for transporting pollen between flowers, bumblebees are often employed as they increase seed set efficiency, fruit quality and yield (Shipp et al., 1994; Portree, 1996; Pressman et al., 1998; Serrano and Guerra-San, 2006).

1.4. Roots and the root environment of hydroponic crops

Primary roles of roots are the absorption and transport of water and nutrients, anchorage of the plant to the growth substrate, synthesis of the phytohormone cytokinin and the release of exudates into the rhizosphere
(reviewed in Nielson, 1974; Fitter, 1996; Gregory, 2006). Cytokinins are produced mainly in root apical meristems and other sites of synthesis include leaves, seeds and shoot apical meristems (Lethan, 1994; Kakimoto, 2003). Cytokinin effects include promotion of cell division and cell enlargement, leaf expansion, leaf greening and delayed leaf senescence (Arteca, 1996).

The rhizosphere, the zone under direct influence of root processes such as exudation and gas exchange, is a core environmental component for PRR epidemics and management in hydroponic crops. Rhizospheres differ substantially depending on whether the crop is grown without a rooting substrate (i.e. in flowing or static nutrient solution only), in a substrate (rockwool, peat or sand) or in a natural soil. The rhizoplane (or root surface) and the endo-rhizosphere (superficial layers of root cells) are also considered by some authors as components of the rhizosphere (Sivasithamparam, 1998; Barea, 2005).

A multitude of abiotic and biotic factors influence the activities and interactions among rhizosphere microbes and plant roots. In contrast to the rhizosphere of crops grown in many natural soils, those in hydroponic systems initially lack large and diverse microbial populations. However, microbial densities often build up substantially during crop growth, in part in response to rhizodeposition (Whipps and Lynch, 1985; Lynch and Whipps, 1990; Gregory, 2006). Rhizodeposition refers to the loss of organic compounds from living roots into the surrounding environment that include secretions, lysates from root cells or whole roots, exudates and gases (Lynch and Whipps, 1990; Pliego et al., 2011). Examples of these substances are amino acids, organic acids, sugars,
enzymes, phenolic intermediates, vitamins, plant hormones, CO₂ and mucilage (Lynch and Whipps, 1990; Graham, 1998; Uren, 2001; Gregory, 2006; Pliego, 2011). The amount of photosynthetically fixed carbon that is transported to roots is generally 30-60% and up to 90% of this carbon is released from the root through rhizodeposition and respiration (reviewed in Lynch and Whipps, 1990).

Rhizodeposition generally increases with age of the roots of a crop, and when diseases such as PRR develop (Lynch and Whipps, 1990; Graham, 1998; Nihorimbere et al., 2011). Many rhizodeposits are important food sources for rhizosphere microbes and some attract microorganisms, including pathogens, to roots (Bais, 2006; Nihorimbere et al., 2011; Pliego, 2011). Flow rates and turbulence of nutrient solutions and surface properties of the rooting medium influence the movement of root exudates and dispersal of microbes, including zoospores of *P. aphanidermatum*.

1.5. Epidemiology and symptoms of Pythium root rot in hydroponic sweet pepper in Ontario

1.5.1. The pathogen

*Pythium aphanidermatum*, the principal *Pythium* species associated with PRR in hydroponic peppers in Ontario, also causes root rot in a large range of other host plants and is widely distributed in hydroponic crops around the world (Ben-Yephet and Nelson, 1999; Sutton et al., 2006). *Pythium* species are classified as oomycetes, members of which were formally grouped in the Kingdom Fungi, but are now commonly described as fungal-like and assigned to
the Chromista (=Straminipila) Kingdom which also includes photosynthetic algae and diatoms (Cavalier-Smith, 1998; Sogin and Silberman, 1998). Primary characteristics that distinguish the Chromista from Fungi include cell walls mainly composed of cellulose as opposed to chitin, rare synthesis of sterols (fungi synthesize ergosterol), the presence of two morphologically-different flagella (fungi have either one or none) and diploid nuclei (not haploid) (Cavalier-Smith, 1998; Sogin and Silberman, 1998).

1.5.2. Inoculum sources

Sources of initial (primary) inoculum of *P. aphanidermatum* in root rot epidemics include contaminated portions of the hydroponic system (e.g. tubing, pipes and the greenhouse floor), soil, dust, infected transplants introduced from other greenhouses and irrigation water (Stanghellini and Rasmussen, 1994; Sánchez et al., 2001; Sutton et al., 2006; Stewart-Wade, 2011). There are reports that insects including shore flies (*Scatella stagnalis*) may vector oospores of *P. aphanidermatum* (Goldberg and Stanghellini, 1990).

1.5.3. Survival of *Pythium aphanidermatum* in hydroponic systems

Oospores are the main survival structures of *P. aphanidermatum* in greenhouses. The oospores normally form inside infected roots and can survive for several months to years in fragments of dead roots and rhizo-deposited materials, in the free-state in troughs, used rooting substrate and plumbing components of the hydroponic systems (Funck-Jensen and Hockenhull, 1983;
Martin and Loper, 1999; Sutton et al., 2006). The oospores are globose, approximately 23 µM in diameter, thick walled (1-2 µm), and resist adverse environmental conditions such as desiccation, extreme temperatures, and agricultural chemicals including fungicides (Plaats-Niterink, 1981; Martin and Loper, 1999). Oospores of *Pythium* spp. exhibit constitutive (inherent) dormancy, but become germinable under the influence of pH, age, root exudates, water, temperature and other variables (Adams, 1971; Martin and Loper, 1999).

1.5.4. Initial and subsequent inoculum

Oospores may germinate to form germ tubes which may directly infect roots. Oospores may also produce sporangia that bear thin-walled vesicles from which zoospores are released (Agrios, 2005). These zoospores are considered a principal form of initial (or primary) inoculum in PRR epidemics. Zoospores subsequently produced on infected roots in sporangia and associated vesicles are the main form of subsequent (or secondary) inoculum in epidemics (Plaats-Niterink, 1981; Rey et al., 1997; Sutton et al., 2006). A single sporangium may produce in excess of 100 zoospores that are released into the aqueous environment when the vesicles burst (Agrios, 2005). Mature zoospores of *P. aphanidermatum* are approximately 14 µm long and 9 µm in diameter (Plaats-Niterink, 1981). New generations of zoospores can be produced within as little as 24 hours in infected roots to initiate new and overlapping infection cycles in the roots (Johnstone, 2001).
1.5.5. Pathogen dispersal

Closed re-circulating systems are inherently conducive to epidemics of PRR. Zoospores, the main dispersal propagule of *P. aphanidermatum*, are detectible in nutrient solutions during most stages of PRR epidemics (Stanghellini et al., 1988; Zinnen, 1988; Stanghellini and Rasmussen, 1994). Zoospores are self-motile by means of flagella, primarily in non-turbulent nutrient solution. Under conditions of turbulence or vibrations, zoospores quickly lose their flagella so become immobilized and unable to locate and infect roots (Holderness and Pegg, 1986; Hardham, 1992). Passive dispersal in flowing or turbulent nutrient solution is the main means of dispersal to downstream plants in hydroponic crops. Oospores and hyphal fragments associated with rotted roots have occasionally been reported to serve as dispersal propagules (Martin and Loper, 1999; Green and Jensen, 2000; Owen-Going et al., 2003).

1.5.6. Root infection and colonization by *P. aphanidermatum*

Zoospores are also the most important infection propagule of *P. aphanidermatum*. Zoospores are attracted to roots primarily through chemotaxis towards compounds in exudates; however electrotaxis (attraction to electrical fields) has also been reported (Jones et al., 1991; Hardham 1992; Morris and Gow, 1993; Martin and Loper, 1999; Van West et al., 2002; Appiah et al., 2005). Zoospores are attracted especially to zones of root elongation, root caps, root hairs, and wounded epidermal cells (Royle 1963; Endo and Colt, 1974; Gold and Stanghellini, 1985; Hardham, 1992; Deacon and Donaldson, 1993; Zhou and
Paulitz, 1993). Zoospores can infect young roots in plants of any growth stage in which the cells are undifferentiated and lack layers of pathogen-resistant lignin and suberin as found in older roots (Zhou and Paulitz, 1993; Kamoun et al., 1999; Martin and Loper, 1999; Agrios, 2005).

After dispersal to roots, zoospores withdraw their flagella, adhere to a roots' surface and encyst (Endo and Colt, 1974). Within 15 minutes, an encysted zoospore may germinate to form a germ tube that directly penetrates the root epidermal cells (Gold and Stanghellini, 1985; Stanghellini and Ramussen, 1996). Factors influencing zoospore activities including encystment were considered in several reviews including those by Appiah et al. (1992) and Hardham (1992).

Mycelia of *Pythium* spp. generally grow intracellularly and intercellularly within young roots, reaching cortical cells in the elongation zone within 24 hours following infection and may continue inwards and colonize the vascular system within 3 days; colonization of older roots is generally limited to the cortex (Endo and Colt, 1974, 1991; Rey et al., 1997).

1.5.7. Symptoms of Pythium root rot in hydroponic sweet peppers

Typical symptoms of PRR in sweet peppers are wilting of the foliage and root browning. Initial infection of roots by *P. aphanidermatum* is biotrophic (i.e. the pathogen feeds on living root tissue) during which stage roots may appear healthy, whitish and symptomless and the leaf canopy normally remains green and conveys the impression that the plants are in good health. The transition from the biotrophic to a necrotrophic (root browning, destructive) stage of
infection is believed to be triggered by an elicitor called NLP$_{Py}$ which was formerly known as the *P. aphanidermatum* necrosis inducing elicitor, PaNie (Veit et al., 2001; Kamoun, 2006; Küfner et al., 2009). This elicitor is a necrosis- and ethylene-inducing peptide 1 (Nep1)-like protein that acts both as a virulence factor and trigger of plant defense responses capable of initiating necrosis and other immunity responses including enhanced synthesis of reactive oxygen species (ROS, considered later) and phenolic compounds (Veit et al., 2001; Qutub et al., 2006; Küfner et al., 2009). In hydroponic sweet pepper, inoculation with *P. aphanidermatum* increased the concentration of the phenolic compounds 4-hydroxybenzoic acid and vanillic acid in roots and in the nutrient solution (Owen-Going et al., 2008, 2011). The NLP$_{Py}$ elicitor is a pore-forming phytotoxin that creates lesions in cell plasma membranes through cytolysis. Plants are able to recognize the membrane degradation processes caused by NLP$_{Py}$, in contrast to the elicitor itself (Quotub et al., 2006; Küfner et al., 2009).

It has been reported that leaves of peppers, lettuce and other crops inoculated with *P. aphanidermatum* often become darker green compared to those of non-inoculated plants (Johnstone, 2001; Sutton et al., 2006). The necrotrophic phase of PRR is characterized by root tip browning, progressive browning of the entire root system and concomitant cell death, collapse and fragmentation of the roots. When *P. aphanidermatum* has caused extensive damage to roots, leaves become chlorotic (yellow), wilted and may fall and the growth of shoots and roots is reduced (Stanghellini and Rasmussen, 1994; Khan et al., 2003; Sutton et al., 2006). Johnstone et al. (2005) reported that *P.*
*aphanidermatum* reduced whole-plant net carbon exchange rates in peppers and caused a loss of 28% in cumulative carbon gain within seven days of inoculation resulting in a reduced expansion rate of the leaves.

1.5.8. Epidemics of Pythium root rot

Pythium root rot is a polycyclic disease in which multiple infection cycles (i.e. infection, colonization, production of secondary inoculum, dispersal of secondary inoculum and new infections) occur during the crop season. Progress of PRR (e.g. root browning) is usually sigmoidal. Disease progress can be extremely rapid when conditions are favorable, especially in vegetative stage peppers, but slows down as available healthy tissue or other factors become limiting. Explosive disease increase is favoured by factors such as the dense population and monoculture of susceptible host plants, rapid and abundant reproduction and dispersal of the pathogen and conditions in the root zone that are conducive to disease such as high temperature, low DO levels and low microbial density and diversity (Stanghellini et al., 1988; Zinnen 1988; Sutton et al., 2006). There are conflicting reports concerning a significant impact of nutrient solution pH on mycelium growth in PRR epidemics (Dimova et al., 2010).

1.5.9. Effects of temperature on Pythium root rot epidemics

Numerous anecdotal reports and observations by growers, advisory personnel, and the research group of JC Sutton at the University of Guelph have associated periods of hot weather and high greenhouse temperatures with abrupt
increases in severity of PRR in hydroponic peppers in Ontario greenhouses (Sutton et al., 2006). High temperature episodes, especially in the root zones of crops, frequently evoke abrupt increases in PRR and serious yield losses in commercial peppers. Root-zone temperatures are commonly high for several hours each day when warm weather prevails and when crop rooting media are exposed to direct sunlight (Sutton et al., 2006). Cooling the root zone as a remedial measure is generally not practical or cost-effective in Ontario greenhouses with re-circulating nutrient solution. Nutrient solutions are in some instances oxygenated when root-zone temperatures are high because the concentration of DO often becomes sufficiently low to favor rapid development of PRR in peppers and other hosts (Chérif et al., 1997; Zheng et al., 2000; Sutton et al., 2006).

Observations under controlled conditions have confirmed that root rot caused by *P. aphanidermatum* is more severe in peppers and other hosts when the temperature is high compared with cooler conditions (Sutton et al., 2006). In hydroponic peppers, root browning (necrosis) increased slowly or not at all at 18-22°C, but rapidly at 28-30°C (Owen-Going et al., 2008). In chrysanthemum grown with constant root-zone temperatures, root browning increased with increasing temperature from 20 to 32°C (Liu et al., 2007). In tomato, spinach, cucumber, soybeans, rye, sugar beets and other hosts, root browning was severe when root-zone temperatures were moderate or high (23-27°C or 35°C), but mild at lower temperatures (Thomson et al., 1971; Plaats-Niterink, 1981; Bates and Stanghellini, 1984; Gold and Stanghellini, 1985; Martin and Loper, 1999; Panova
et al., 2004; Sutton et al., 2006). Under low temperatures (e.g. 15-20°C) inoculated roots may be colonized but symptomless (Littrell and McCarter, 1970; Sutton et al. 2006). In general, high temperatures reported as favorable for severe PRR were similar to those encountered in the root zone of peppers during warm or hot conditions in commercial greenhouses in Ontario. Enhanced disease severity at higher temperatures may be related in part to increased ability of the pathogen to infect the host and to shifts in interactions between pathogens and other organisms in the root zone (Martin and Loper, 1999). However, as Martin and Loper (1999) pointed out, host susceptibility to root rot is also undoubtedly influenced by temperature. Thus high temperature may predispose roots of peppers and other crops to PRR by increasing root susceptibility to *P. aphanidermatum*.

1.6. Temperature in relation to hydroponic sweet peppers

Temperatures of the aerial environment and root zone during the production of hydroponic pepper crops in Ontario greenhouses are usually maintained in the range of 20 to 28°C and seldom below 18°C. During periods of hot or sunny weather the air temperature may exceed 40°C, despite practices to minimize temperature increase such as by the use of shades, evaporative cooling, and ventilation. During such periods the root-zone temperature can remain above 30°C and reach as high as 35°C for several hours during the day (Sutton et al., 2006; Liu et al., 2007). In general, various growth and physiological responses of sweet peppers relate more closely to mean temperature values for
24-hour daily periods than to temperature differences during the day and at night (Bakker and Uffelen, 1988).

Growth, development and yield of hydroponic sweet peppers are optimal at an aerial temperature between 18 to 23°C and are normally reduced above 32°C (Poulos, 1993; Saha et al., 2010). The optimum root-zone temperature for commercial production from seed germination to the development of first true leaves is about 23-26°C, but is lower (20-23°C) for subsequent vegetative growth (Bakker and Uffelen1989). When peppers were grown in a substrate at various constant root zone temperatures, leaf area was found to be greatest at 24°C but the number, area and dry weight of leaves decreased as temperature increased above 25°C (Dodd et al., 2000). Flowering has been shown to be reduced when the root zone is above 30°C while fruit yield is highest when the temperature is maintained at 30°C (Gosselin and Trudel, 1986).

1.7. High temperature predisposition of plants to disease.

Plants are frequently subjected to various abiotic and biotic environmental stress conditions that can predispose them to disease (Yarwood, 1959; Schoeneweiss, 1975; Lockwood, 1988; Jarvis, 1992; Boyer, 1995). Environmental predisposition can be defined as an increased susceptibility of plants to disease induced by environmental stress factors acting prior to infection by the pathogen. The existence of threshold levels for predisposition has been demonstrated by Schoeneweiss (1975) in woody plants. It has also been
reported that stress responses can be reversible or irreversible depending on the magnitude of the stressor.

High temperature has been reported to predispose numerous kinds of plants to disease including grapefruit to Scythalidium wilt (Sadowsky, 2007), greenhouse tomato to tomato spotted wilt virus (Mitidieri et al, 2001), coffee to anthracnose (Chen et al., 2003) and chrysanthemum to Phytophthora root rot (MacDonald, 1991).

Low levels of DO and accumulated phenolic compounds in the root zone of hydroponic crops also predispose roots to attack by *Pythium* so may potentially interact with effects of high temperature. Root rot symptoms in tomato were more severe under conditions of moderate (5.8-7.0%) or low (0.8-1.5%) DO levels than at high levels (11-14%), but it was not determined whether the influence of reduced oxygen was through increasing susceptibility of the host, or through effects on the pathogen or the host-pathogen interaction (Chérif et al., 1997). In hydroponic sweet pepper addition of several phenolic compounds, including 4-hydroxybenzoic acid and vanillic acid, induced toxicity in plants and predisposed their roots to browning induced by *P. aphanidermatum* (Owen-Going et al., 2004, 2011).

Quantitative relationships of an environmental stress factor with plant stress responses and host predisposition to disease have not been reported in most instances, but were reported for low-intensity light in relation to grey mold caused by *B. cinerea* in black spruce and tomato (Zhang and Sutton., 1994; Shafia et al., 2001). Quantitative effects of high temperature on root rot
susceptibility could be investigated and quantified by exposing plants to high
temperature episodes before the roots are inoculated with the pathogen, and
thereby satisfying the requisite conditions to demonstrate host predisposition to
disease (Colhoun, 1979). Quantitative relationships of high temperature episodes
to root rot susceptibility in peppers has yet to be explored, but has potential value
for predicting PRR and the need for remedial measures.

1.8. Physiological responses of hydroponic plants to high root-zone
temperature

High root zone temperature is a principal stress factor in hydroponic crops
in commercial greenhouses. Heat stress shifts the balance of phytohormones,
reduces the activity of most proteins and induces production of heat shock
proteins (HSPs) that mediate repair processes of proteins damaged by high
temperature. Levels of the hormones such as abscissic acid, ethylene and
jasmonic acid, which play active roles in thermotolerance as well as serve as
signalling molecules during plant stress, increase following high temperature
episodes (reviewed in Wahid et al., 2007). Physiological changes of roots in
response to high temperature stress are similar to those that occur during root
senescence (Taiz and Zeiger, 2006).

High root-zone temperature can lead to decreased root and shoot growth,
lower water and nutrient uptake and increased transpiration and respiration rates
(Wahid et al., 2007; Falah et al., 2010). Demand for oxygen by roots in the
nutrient solution generally increases as temperature increases and Jarvis (1992)
proposed that there is a ten-fold increase in the DO requirement of hydroponic crops under stress as compared to non-stressed crops. Respiration rates of roots in hydroponic crops have been reported to double with each 10ºC increase up to 30ºC (Morgan 2001).

High root-zone temperature may lead to reduced DO levels (hypoxia) in the nutrient solution. For example, at 10, 20, and 30ºC, respectively, Morgan (2001) reported DO levels of 13, 9-10, and 7 mg O₂ L⁻¹, respectively, for fully aerated nutrient solutions. Under conditions of prolonged and insufficient root-zone oxygen plants can suffer from wilting, reduced absorption of minerals and water by roots, reduced photosynthesis, reduced shoot and root growth, and increased ethylene accumulation that leads to root cell damage (Drew and Lynch, 1980; Chérif et al., 1997;). Separation of the effects of high temperature and hypoxia in the nutrient solution is often difficult (reviewed in Sutton et al., 2006).

1.9. Plant stress

Plant stress can be interpreted as internal and external conditions that adversely affect plant growth and development. Plants rarely grow under optimum conditions and continuously respond and adapt to numerous concurrent stresses. For each environmental variable there is an optimal range for plant growth above which and below which stress responses of the plant become increasingly more severe. Stress responses of plants are influenced by the intensity, frequency and duration of the stress factor(s) as well as the genetic
background, developmental age and history of the plant. Plants might not recover following extreme or prolonged stress conditions. A stress factor may affect disease development via an effect on the pathogen, host susceptibility and host-pathogen interaction.

1.10. Responses of plants to abiotic and biotic stress factors

A general response to environmental stressors is rapid generation and accumulation of ROS that include singlet oxygen, hydrogen peroxide, superoxide anions, and hydroxyl radicals (Dat et al., 2000; Mittler, 2002). Oxidative stress is defined by Bartosz (1997) as “a shift of the balance between pro-oxidative and anti-oxidative reactions in favor of the former”. At normal levels ROS play roles as signalling molecules and regulators of plant growth and development. However, at high levels, ROS can cause DNA degradation, increased lipid peroxidation and proteolysis (Dat et al., 2000; Mittler, 2002). Increases in ROS induced by high temperature induce increased fluidity and permeability of membranes and decreased integrity, stability and transport functions in membranes (Sutherland, 1991; Wahid, 2007). The degree of membrane damage depends on the intensity and duration of temperature stress (Falah et al., 2010; Wahid et al., 2007). Mechanisms that scavenge excess ROS include the production of antioxidants (e.g. ascorbate, glutathione, Vitamin E) and antioxidant enzymes (e.g. superoxide dismutase, catalase, and peroxidase) and phenolic products (reviewed in Mittler, 2002).
Various environmental stressors, including high temperature and pathogens such as *P. aphanidermatum*, can enhance the expression of phenylalanine ammonia lyase (PAL), the principal enzyme of the phenylpropanoid pathway. Numerous phenylpropanoid compounds are involved in the mediation of stress responses including lignin, suberin and other polyphenolic compounds which accumulate in cell walls (Hahlbrock and Scheel 1989; Dixon and Paiva, 1995). Root infection by *P. aphanidermatum* activates PAL and promotes the accumulation of phenolic compounds associated with inoculated roots, several of which were reported to contribute to root browning (Owen-Going et al., 2011).

The hormones abscissic acid, ethylene, cytokinin and auxin synthesized in the isoprenoid pathway (terpenes) mediate stress responses through the regulation of membrane fluidity, photosynthesis, growth and respiration and other mechanisms (reviewed by Sopher, 1998). In general, synthesis of abscissic acid and ethylene increases under stress conditions and synthesis of cytokinins and auxin decrease. Stomatal aperture and increased membrane permeability are regulated by abscissic acid. Ethylene accelerates abscission and senescence and can stimulate or inhibit shoot and root growth (Jing et al., 2005; Pierik et al., 2006). Under stress conditions, increased ethylene in plants may cause reduction of growth (reviewed in Esitken, 2011). Cytokinins contribute to cell membrane integrity, cell enlargement, cell division and delayed senescence. Indole acetic acid and other auxin compounds regulate cell elongation and membrane lipid composition and the initiation and development of roots,
1.11. Management of Pythium root rot in hydroponic systems

Practices for managing PRR caused by *P. aphanidermatum* in crops grown in hydroponic systems were summarized in several reviews including Sutton et al. (2006) and Stewart-Wade (2011). An integrated approach that utilizes all methods available to growers (cultural, biological and chemical) is preferred. These measures do not fully control PRR, and resistant pepper cultivars have not been developed. Use of synthetic pesticides in hydroponic peppers is greatly restricted in Ontario and registration is limited to the preventative fungicide Previcur® N (propamocarb hydrochloride) produced by Bayer Environmental Science for minor use or emergency use (OMAFRA, 2011). A concern with use of this fungicide is phytotoxicity because of the lack of buffering in nutrient solutions and development of resistance by the pathogen (Utkhede et al. 2000; Paulitz and Bélanger, 2001). From an epidemiological perspective, disease management practices can achieve two principal effects, first to prevent or reduce the amount of initial inoculum and second to reduce the rate of increase in severity of the epidemic (Fry, 1982; Bergamin Filho and Amorim, 1996). Epidemiological knowledge, such as the polycyclic nature of PRR, also emphasizes that root protection should begin early in crop growth (e.g. at the seedling stage) and continue throughout the crop cycle. A key goal is to prevent or delay the transition from biotrophy to necrotrophy, since the latter is damaging to the host.
Excluding *P. aphanidermatum* from a greenhouse reduces the initial inoculum and requires the use of effective sanitation measures before, during and after each crop cycle. The goal is to destroy or remove the pathogen in sources within the greenhouse such as crop residues, growing media, plastic sheeting, hardware components of hydroponic systems (containers, pipes, tubing, troughs etc.) and interior parts of greenhouse structures (floors, walls, benches etc.). Destruction of inocula in these sources is often achieved by steam pressure cleaning, chemical sterilants and temporary acidification of the nutrient solution (pH 2.0 or 3.0). Disinfestation of water used for nutrient solution, use of pathogen-free transplants and planting media, use of screening on ventilators to exclude insect vectors and disinfestation of footwear also help exclude the pathogen (Hong and Moorman, 2005; Sutton et al., 2006).

Reducing the rate of increase of PRR may involve disinfestation of the nutrient solution as it circulates *outside of* the crop and suppression of the pathogen mainly *in the root-zone* of the crop. Inside the crop, disinfestation techniques include the use of heat pasteurization, ultraviolet irradiation, ultrafiltration (membrane filtration), mechanical and biological filtration, biosurfactants, ozone treatment, chlorination, and activated hydrogen peroxide. Details and limitations of some of these measures have been discussed extensively (Menzies and Belanger, 1996; Stanghellini et al., 1996; Van Os et al., 1999; Sutton et al., 2006; Stewart-Wade, 2011). The effectiveness of measures to kill or inactivate zoospores as they circulate outside of the crop is questionable since zoospores are almost exclusively produced in the root zone and rapidly
attach to roots. Research by Owen-Going (2002) indicates that few or no zoospores in the effluent from the root zone of the crop are able to endure dispersal through the mixing tank and back to the crop. Anecdotal evidence in Ontario and Europe indicate that increased compartmentalization of hydroponic crops such that few, rather than many, share the same nutrient solution as it circulates in the crop has reduced the frequency and severity of PRR epidemics.

Measures aimed at maintaining plant health by minimizing plant stress from environmental factors such as high temperature, low level of DO in the nutrient solution, low intensity light and overcrowding of plants can be advantageous for managing PRR (Sutton et al. 2006; Corrêa et al., 2012). When the root-zone temperature is high, addition of cool water to the nutrient solution, increasing the flow rate and adding oxygen to the nutrient solution can be used to maintain DO levels that are adequate for plant health (Sutton et al., 2006). Another approach to maintain plant health is the use of beneficial bacteria that suppress disease. Several beneficial bacteria can enhance plant growth as well as to suppress PRR in hydroponic environments (Martin and Loper, 1999; Khalil and Alsanius 2011; Corrêa et al., 2012). Advantages of using beneficial bacteria as compared to chemical and other disease management measures in hydroponic systems were reviewed (Sutton et al., 2006; Corrêa et al., 2012).
1.12. The use of beneficial bacteria to suppress Pythium root rot in hydroponic crops

Greenhouse hydroponic systems are particularly suitable for the use of introduced beneficial bacteria because they usually provide the necessary nutrients and ideal environmental conditions for microbial growth and reproduction. Environmental parameters in greenhouses such as root-zone pH, nutrient composition and temperature can be manipulated to optimize their biocontrol capabilities (Sutton et al., 2006). The density and diversity of natural microbial populations in the root zone are generally low at early stages of crop growth and progressively increase over time (Zheng et al., 2000; Paulitz and Bélanger, 2001). For example, Zheng et al. (2000) detected $10^5$ bacterial colony forming units (CFU) mL$^{-1}$ nutrient solution just after transplanting and $10^8$-$10^9$ CFU mL$^{-1}$ 10-14 weeks later in root zones of hydroponic cucumbers grown in small scale trough systems. Microbial diversity has also been shown to increase significantly in the root-zone of hydroponic crops a few weeks following transplantation, as demonstrated by Postma et al. (2000) in cucumbers grown in rockwool. The abundance of organic materials such as plant exudates and sloughed off root cells increases as a crop develops and serve as nutrients for the microbial populations (Postma, 2009). *P. aphanidermatum* incidence and PRR increases rapidly when levels of microbes that are capable of competing with the pathogen are low, thus microbial protection of the roots of hydroponic crops is usually low during a substantial portion of the crop season (Stanghellini and Rasmussen, 1994; Khan et al., 2003; Liu et al., 2007).
Studies suggest that the effects of beneficial microbes are often more pronounced under conditions of abiotic and biotic stress such as low iron, high salinity, high temperature and high pathogens than under non-stress conditions (reviewed in Pliego et al., 2011). Increased marketable yield and size of greenhouse tomato attributed to inoculation with *P. fluorescens* strain 63-28 was greater when light and temperatures (low) were suboptimal as opposed to when plants were grown under optimal conditions. Rankin and Paulitz (1994) reported that marketable yield of hydroponic cucumbers grown in rockwool slabs and treated with *Pseudomonas corrugata* strain 13 or *Pseudomonas fluorescens* strain 15 followed by inoculation with *P. aphanidermatum* was increased 88% in a spring crop and 600% in a fall crop. The latter crop was exposed to higher (25-30°C) temperatures in the root zone and suffered more severe PRR.

Many beneficial microbes belong to a group of non-pathogenic free-living root-associated bacteria referred to as plant growth promoting rhizobacteria (PGPR) (Kloepper et al., 1980; Saharan and Nehra, 2011). Kloepper and Schroth (1978) defined PGPR as “rhizobacteria that exert beneficial effects on plant development”. Root colonization (rhizosphere competence) is fundamental for PGPR to exert their beneficial effects and lack of these desired effects is often associated with inadequate root colonization (Benizri et al., 2001). These bacteria can promote plant growth directly, in the presence or absence of a pathogen, through mechanisms including synthesizing phytohormones and enhancing nutrient uptake (Esitken 2011; Pathma et al., 2011; Pliego, 2011). Glick et al. (1998) suggested that plant growth promotion by PGPR could be
achieved through their capacity to lower ethylene level in a plant, which would prevent the deleterious effects of some environment stressors (Esitken 2011). The PGPR may also stimulate plant growth indirectly by changing the microbial balance in the rhizosphere in favour of beneficial microorganisms (reviewed in detail in Esitken (2011) and Pathma et al. (2011).

A number of investigations suggest that PGPR can suppress or decrease pathogen populations through various mechanisms (which are not necessarily mutually exclusive). These mechanisms include rapid occupation of, and persistence in, the rhizosphere and root surface competition for essential nutrients, niche exclusion, antibiosis, enzymatic cell wall degradation and induced systemic resistance, or ISR (Campbell, 1989; Whipps, 1997; Chen et al., 1998; Pathma et al., 2011).

Kloepper et al. (1992) defined ISR as “a process of active resistance dependent on the host plant’s physical or chemical barriers, activated by biotic or abiotic agents (inducing agents)” that protects the plant systemically when applied at one location on a plant. Activation of ISR occurs only after the exposure to a stressor and normally reduces the rate of disease development and severity (reviewed in Van Loon et al., 1998; Conrath et al. 2002). Key signalling molecules in ISR include the phytohormones ethylene and jasmonic acid and ROS (Chapin, 1991; Kotak et al., 2007). It is important to note that only non-pathogenic PGPR induce ISR in a host and that the level of ISR decreases with time, although the effects may last for weeks (Liu et al., 1995).
Reports indicate that induced systemic resistance (ISR) is an overwhelmingly important mechanism of plant protection by strains of the fungal agent *Trichoderma harzianum* T-22 (Harman et al., 2004) but similar comprehensive studies of mechanisms of antagonism have not been reported for PGPR.

Several PGPR, including *Pseudomonas* spp., exhibit cell-cell communication and synchronization of cell populations to produce signals for the expression of specific genes involved in processes such as colonization and antibiosis. One signalling system is referred to as quorum sensing which has been defined by Bais (2006) as “the density dependent mechanism used by many bacteria to regulate gene expression in a coordinated manner” in which “density” refers to the bacterial population. Quorum sensing involves the production of signalling compounds (autoinducers) such as N-acylhomoserine lactones, which at threshold levels, leads to induction of gene expression. For example, a threshold initial inoculum level of PGPR is required to significantly stimulate plant growth (Teplitski et al., 2000). Quorum sensing is used by bacteria to coordinate the formation of biofilms on a root surface in which groups of microbes are attached to each other and as a population regulate processes involved in defense, colonization, signalling, survival, attraction and attachment (Davey and O’Toole, 2000; Morris and Monier, 2003). It is important to understand that populations, not individual organisms, are required for good disease suppression (Pliego, 2011). For example, production of various metabolites by PGPR including antibiotics is regulated by quorum sensing (Loh
et al. 2002). Detailed information on quorum sensing is available in numerous reviews (Bloemberg and Lugtenberg, 2001; von Bodman et al., 2003; Diggle et al., 2008). Obstruction of quorum sensing is referred to as quorum quenching (Zhang, 2003).

1.13. *Pseudomonas chlororaphis*

*Pseudomonas chlororaphis*, also referred to as *P. aureofaciens* (Johnson and Palleroni, 1989) or *P. fluorescens* (Paulitz and Bélanger 2001), is of particular interest for use as a beneficial microbe because of PGPR properties as described above. Like some other *Pseudomonas* spp., it enhances plant growth and provides plants with protection against root-infecting pathogens and environmental stresses (Bakker et al., 2003; Haas and Keel, 2003; Mercado-Blanco et al., 2007; Weller, 2007). This bacterium has a wide host range, is non-phytopathogenic, saprotrophic, gram-negative, rod-shaped and motile (Haas and Défago, 2005). Cells of *P. chlororaphis* are about 0.5-1 X 1.5-5 µm in size. *Pseudomonas chlororaphis* is attracted to roots by chemotaxis and primarily penetrates root tissue through wounds and natural openings such as sites of emergence of lateral roots. It is able to quickly establish colonies epiphytically on root surfaces and endophytically within the root cap, epidermis and cortex (Compant et al., 2005; Botelho and Mendonça-Hagler, 2006).

*Pseudomonas chlororaphis* promotes plant growth and suppresses PRR in sweet pepper and other crops under a range of environmental conditions in hydroponic crops (Corrêa et al., 2010; Corrêa et al., 2012). McCullagh et al.
(1996) reported increased growth and yield of cucumber plants inoculated with *Pseudomonas fluorescens* 63-28 in the presence and absence of *Pythium aphanidermatum*. Cytokinins have been reported to be produced by *P. chlororaphis* 63-28 (EPA, 2011) which is believed to promote plant growth by increasing nutrient availability in the rhizosphere and stimulating plant growth (Vessey, 2003).

Evidence indicates that suppression of PRR caused by *P. aphanidermatum* by strains of *P. chlororaphis* is mediated by several mechanisms including antagonism, competition for infection sites and nutrients, antibiosis and ISR. *P. chlororaphis* Tx-1 significantly reduced root colonization by *P. aphanidermatum* in hydroponically-grown sweet pepper (Chatterton, 2004) and chrysanthemum (Liu et al., 2007). This bacterium also reduced zoospore encystment and germination on cucumber roots (Chen et al., 1998; Zhou and Paulitz, 1993). Zheng et al., (2000) demonstrated that *P. chlororaphis* strain JZ-24 suppressed *P. aphanidermatum* by destroying root mucilage, thus eliminating a source of nutrients for the pathogen, in addition to competing for infection sites. *Pseudomonas chlororaphis* can also suppress *Pythium* through the secretion of several antimicrobial metabolites and increased hydrolytic enzyme production. Several strains of *P. chlororaphis* produce phenazine antibiotics (Chin-A-Woeng et al., 2000; Maddula et al., 2006), however phenazine production by strain 63-28 (used in the present investigations) has not been reported. Chen et al. (2000) reported that *P. chlororaphis* induced several phenolic intermediates such as PAL, peroxidase, and polyphenol oxidase in cucumber.

The background information on the epidemiology and control of PRR, summarized above, suggests that management of the disease might be improved given a quantitative understanding of high temperature episodes in relation to PRR and of the ability of *P. chlororaphis* to suppress PRR when high temperature episodes occur. While many authors have noted that PRR is more severe under conditions of high temperature, relationships of the duration, frequency and intensity of high temperature episodes to root rot severity are not known. Further, it is not clear whether high temperature predisposes plants to PRR, and if this is true, what the quantitative relationships are between high temperature episodes and predisposition. Quantitative information on high temperature in relation to PRR has potential applications in managing PRR such as through timing applications of beneficial microbes such as *P. chlororaphis* 63-28 in the root zone. Strain 63-28 was found to be superior in its disease control activity among more than 4000 bacterial strains isolated from Canadian soils (Kloepper et al., 1988), and previous reports suggest that this strain justifies further investigation for dual activity to enhance productivity and suppress PRR in hydroponic crops. Further studies are needed in part to determine the effectiveness of the agent under conditions of high temperature and in plants stressed to various degrees by high temperature. Taken together, the data may provide a rationale for predicting abrupt increases in disease associated with high temperature episodes and thus opportunity for timely applications of *P. chlororaphis* or other control measures in commercial crops. Sweet pepper was
chosen as the host based on the importance of the crop and of PRR in hydroponic greenhouses in Canada and in view of the substantial platform of epidemiological knowledge accumulated on PRR in this host.

The isolate of *P. aphanidermatum* used throughout this thesis was isolated from a hydroponic cucumber root in south western Ontario by Dr. W.R. Jarvis and referred to as isolate 167, then renamed P6. The identity of isolate P6 was confirmed by C. Sopher using microscopic and molecular techniques at the First International Workshop for the Morphological and Molecular Characterization of the Straminipiles: *Phytophthora* and *Pythium*, held at North Carolina State University in 1994.

1.15. Thesis objectives

The objectives of the present thesis were as follow:

1. To quantify relationships between the duration of high root-zone temperature (33°C) immediately before inoculation and subsequent root colonization by *P. aphanidermatum* and progress of root browning.
2. To quantify effects of delayed inoculation with *P. aphanidermatum* following high temperature treatment on the progress of root browning.
3. To determine relationships of inoculum density of *P. chlororaphis* 63-28 applied in the nutrient solution to progress of root browning caused by *P. aphanidermatum*.
4. To determine the optimum density of *P. chlororaphis* 63-28 inoculum for root rot control.
5. To examine densities of *P. chlororaphis* 63-28 associated with the roots as a function of time after application, root-zone temperature regimes, and inoculation with *P. aphanidermatum*.

6. To examine quantitative effects and interactions of *P. chlororaphis* 63-28, root-zone temperature regimes, and *P. aphanidermatum* on growth parameters of vegetative peppers.

7. To assess leaf expansion as a minimally intrusive means to estimate effectiveness of *P. chlororaphis* 63-28 against root rot.
Chapter 2. Relationships of pre-inoculation high temperature to root browning caused by *Pythium aphanidermatum* in hydroponically-grown sweet pepper

2.1. Abstract

Episodes of high root-zone temperature (33°C) were investigated in relation to susceptibility of sweet pepper to root rot caused by *Pythium aphanidermatum*. Pepper plants were grown in aerated nutrient solution in hydroponic units positioned in temperature-controlled water baths. Root zone temperature was 23°C except during high temperature treatments. Roots were exposed to 33°C for 0 to 216 h, inoculated with *P. aphanidermatum* and assessed at intervals for colonization by the pathogen and for severity of browning (necrosis). The pathogen colonized all roots within 12 h following inoculation. The roots turned brown earlier when exposed to 33°C for more than 9 h prior to inoculation compared to those exposed for 0 to 6 h. Browning developed progressively earlier as the period at 33°C was increased from 9 to 144 h, and was 4 days earlier for exposures of 144 to 216 h compared to 0 to 9 h. Browning was precocious also when inoculation was delayed as long as 216 h following exposure at 33°C for 72 h. I conclude that high temperature predisposes pepper plants to precocious browning caused by *P. aphanidermatum* and that predisposition lasts for at least 9 days.

2.2. Introduction

*Pythium* root rot is a major factor limiting the productivity and profitability of
hydroponically-grown sweet peppers (Capsicum annuum L.) in Ontario (Owen-Going, 2002; Owen-Going et al., 2003). The disease is characterized by root tip browning, expansive browning of the roots, reduced growth rate of the roots and shoots, and during advanced stages of pathogenesis, wilting and yellowing of the foliage (Owen-Going et al., 2003; Johnstone et al., 2005; Sutton et al., 2006). *Pythium aphanidermatum* (Edson) Fitzp. is the principal causal agent of root rot, but several other species of *Pythium* were reported to cause mild root symptoms in hydroponic peppers (Owen-Going et al., 2003). Numerous anecdotal reports by growers, advisory personnel, and our research group leave little doubt that periods of high temperature in hydroponic greenhouses, especially in the root zones of crops, frequently evoke increases in severity of Pythium root rot and serious yield losses in commercial peppers. Temperature of the root-zone is commonly high (e.g. 28 to 35°C) for several hours each day when warm weather prevails and also during cool weather when the rooting media of the crop such as rockwool or coconut fibre (coir) are exposed to direct sunlight (Sutton et al., 2006). Cooling the root zone as a preventative or re-medial measure is generally not feasible or cost-effective in Ontario greenhouses. Until recently root zones were commonly cooled by introducing fresh nutrient solution prepared with cool water, but this method is no longer practiced because of needs to conserve water resources, minimize fertilizer use, and limit disposal of used solutions into the environment. Nutrient solutions are in some instances oxygenated when root-zone temperatures are high because the concentration of dissolved oxygen is often sufficiently low to favour rapid development of root rot associated with *P.*
aphanidermatum and other *Pythium* spp. in peppers and other hosts (Chérif et al., 1997; Zheng et al., 2000; Sutton et al., 2006). Alternative means for controlling root rot in hydroponic peppers includes use of biological control organisms, fungicides, and methods to disinfest the nutrient solution (Corrêa et al., 2009).

Observations under controlled conditions have confirmed that root rot caused by *P. aphanidermatum* is more severe in peppers and other hosts when the temperature is high compared with cooler conditions (Sutton et al., 2006). In hydroponic peppers, root browning (necrosis) increased slowly or not at all at 18-22°C but rapidly at 28-30°C (Owen-Going et al., 2008). In chrysanthemums grown in single-plant hydroponic containers with constant root-zone temperatures, root browning increased with temperature from 20 to 32°C (Liu et al., 2007). In tomato, spinach, cucumber, soybeans, rye, sugar beets and other hosts, root browning was severe when root-zone temperatures were moderate or high (23-27°C or 35°C), but mild at lower temperatures (Thomson et al., 1971; Plaats-Niterink, 1981; Gold and Stanghellini, 1985; Martin and Loper, 1999; Panova et al., 2004; Sutton et al. 2006). Under low temperatures (e.g. 15-20°C) inoculated roots may be colonized but symptomless (Littrell and Mc Carter, 1970; Sutton et al. 2006). In general, high temperatures reported as favourable for severe root rot were similar to those encountered in the root zone of peppers during warm or hot conditions in commercial greenhouses in Ontario. Enhanced disease severity at higher temperatures may be related in part to increased ability of the pathogen to infect the host and to shifts in interactions between pathogens.
and other organisms in the root zone (Martin and Loper, 1999). However, as Martin and Loper (1999) pointed out, host susceptibility to root rot is also undoubtedly influenced by temperature.

Several authors surmised that the greater severity of Pythium root rot under high temperature conditions is due to responses of the host plants to environmental stress factors (stressors), particularly elevated temperature in the root zone or plant canopy and reduced concentration of dissolved oxygen in the nutrient solution (Sutton et al. 2006). The notion that exposure of plants to environmental stress factors increases host susceptibility to root rot has not been conclusively demonstrated. Clearly, the potential exists for the host and pathogen to respond both independently and interactively to high temperature conditions. In the previous reports, the roles of the host and pathogen in mediating effects of high temperature on root rot could not be differentiated because the pathogen (P. aphanidermatum or other Pythium sp.) was present in the root zone at the time of high temperature treatment. Under conditions in culture, mycelial growth of P. aphanidermatum is rapid at 30-40°C and relatively slow at 10-25°C, while zoospore production is abundant at 17-31°C but not at 35°C or 37°C (Plaats-Niterink, 1981; Gold and Stanghellini, 1985). Regardless of the importance of the pathogen as a mediating factor, a clear and quantitative understanding of the role of the host would be advantageous for predicting and managing Pythium root rot. Effects of high temperature on root-rot susceptibility can be investigated and quantified by exposing plants to high temperature episodes before the roots are inoculated with the pathogen, and thereby satisfy the requisite conditions to
demonstrate host predisposition to disease (Colhoun, 1979). Predisposition normally refers to an increased susceptibility of plants to disease brought about by environmental factors acting prior to infection by the pathogen (Jarvis, 1992).

Quantitative information of the frequency, duration, intensity (in °C), and time of occurrence of episodes of high root-zone temperature in relation to predisposition of peppers to root colonization by *P. aphanidermatum* and the development of root browning could provide an effective basis for predicting the risk of rapid disease increase and the need for re-mediation measures.

In a preliminary study, it was found that exposure of the roots of healthy pepper plants to high temperature (28-33°C) for several days immediately prior to inoculation with *P. aphanidermatum* predisposed the plants to increased severity of root rot. The present research was conducted to further investigate high root-zone temperature in relation to predisposition of peppers to *Pythium* root rot. The first objective was to quantify relationships between the duration of high root-zone temperature (33°C) immediately before inoculation and subsequent root colonization by *P. aphanidermatum* and progress of root browning. The second objective was to quantify effects of delayed inoculation following high temperature treatment on the progress of root colonization and browning.

2.3. Material and Methods

2.3.1. Hydroponic pepper plants

Seeds of sweet pepper (*Capsicum annuum*) cv. Cubico (DeRuiter Seeds Inc., Columbus, OH, USA) were germinated in rockwool plugs (2.5 cm X 2.5 cm
X 4.0 cm; Grodan, Roermond, The Netherlands) in plastic trays in a growth room. Plants were watered with de-ionized water during the initial three weeks and with half-strength nutrient solution in the subsequent two weeks. At five weeks after sowing, the pepper plants were transferred to single-plant hydroponic units on a bench in the growth room. Each plant in its rock-wool plug was placed in a 5 cm-diameter mesh pot (Homegrown Hydroponics, Breslau, ON) positioned in a hole made in the lid of a 475 mL white polyethylene container filled with nutrient solution. To exclude light, the container lids were covered with black-on-white plastic, white side up. The containers were arranged in black plastic trays (100 cm x 38 cm x 19 cm deep) on a bench in a growth room. The nutrient solution was prepared with 0.73 g of soluble fertilizer (NPK, 7:11:27; plus microelements; Plant Products Ltd., Brampton, ON, Canada) and 0.48 g Ca(NO$_3$)$_2$ per litre of de-ionized water, and adjusted to pH 5.8. The electrical conductivity (EC) was near 1.5 mS·cm$^{-1}$. The nutrient solution in each unit was continuously aerated by means of aquarium air pumps and plastic tubes (2 mm internal diameter) and replenished as needed. The dissolved oxygen (DO) content of the aerated solutions was monitored with an oxygen meter (model YSI 55, Yellow Springs Instruments Co. Inc., Yellow Springs, OH, USA) and ranged from 5 to 7 mg O$_2$/L during experiments. The nutrient solution temperature normally equilibrated 0.5 - 1.0°C below that of the air temperature of the growth room, which was maintained at 23-24°C. The photoperiod was 16 h and light was provided by fluorescent tubes (115 W Cool White, GTE, Sylvania Ltd, Canada) supplemented with incandescent bulbs. The intensity of photosynthetically active radiation
(PAR) at plant height was 180-200 μmol/m²/s as measured by quantum sensors (Q 3991-4 LI-COR Inc., Lincoln, NE). The relative humidity was maintained at 60-65%. Plants were used for experiments when 7-8 weeks old, 9-11 cm tall and had root systems 8-0 cm long.

2.3.2. High temperature treatment of roots

For investigations of pre-inoculation high temperature of the root zone in relation to root rot, temperature-controlled water baths were positioned on a bench beneath light banks in the growth room. Each bath comprised a 37.8 L plastic tote box with the sides and bottom insulated with foil-covered bubble wrap. Plants in the hydroponic containers were maintained in the baths throughout experiments (Figure 2.1).

Water temperature of unheated baths was near 23°C. For high temperature treatments the water in each bath was heated to 33°C by means of two thermostatically-controlled aquarium heaters (model 100 W Tronic, Rolf C. Hagen, Montreal, QC). Approximately 3 h was required to raise the temperature of the plant nutrient solution from 23 to 33°C. Immediately following high root-zone temperature treatments the aquarium heaters were turned off and the water and nutrient solution cooled to 23°C during 3-4 h. The temperature of the nutrient solution was measured frequently during experiments using calibrated alcohol-in-glass thermometers.
Figure 2.1. Single-plant hydroponic units with pepper plants in a temperature-controlled water bath.
2.3.3. *Pythium* inoculum and inoculations

*Pythium aphanidermatum* isolate P6 from hydroponic cucumber was maintained for up to 12 weeks on roots of living host plants and recovered when needed by incubating pieces of the colonized roots for two days on P₅AR, a *Pythium*-selective agar medium (Owen-Going et al., 2003). To produce zoospores, colonies of the pathogen grown on 20% V-8 medium in Petri dishes were flooded with sterilized de-ionized water after 48 h of incubation and again after 96 h under a critically-controlled temperature regime (Owen-Going et al., 2003). Zoospores released during the second flooding were collected in plastic beakers. Zoospore density in the aqueous suspensions was estimated by vibrating 1-mL samples in microfuge tubes on a Vortex mixer (Fisher Scientific, Toronto, ON, Canada) for 30 s and counting the immobilized spores on a haemocytometer. For inoculations the suspensions were diluted to 5 x 10³ zoospores mL⁻¹ and gently poured into plastic trays. For inoculation, plants were carefully positioned in the trays with all of the roots immersed in the zoospore suspension for 30 min. The plants were returned to their respective hydroponic units immediately after inoculation. Roots of control plants were immersed in sterilized de-ionized water.

2.3.4. Estimation of colonization and discoloration of the roots

To estimate percent roots colonized by *Pythium*, 20 principal roots were removed from each plant and cut into 1-cm-long segments. The segments were surface-disinfested in 1% NaOCl for 30 s and in 70% ethanol for 30 s, rinsed
three times in sterile distilled water and blotted dry. Thirty random segments from each plant were incubated on the P5AR selective medium at 25°C for 48 h and assessed for incidence of *P. aphanidermatum*. For estimating root discoloration, each plant was held with the roots above the nutrient solution and its root system was examined for about 15 s with the aid of hand lens (10X magnification). The percent of root tips and of total roots that were discoloured were estimated using an equal-increment scale of 0-10 (0 = 0%, 1 = 1-10%, 2 = 11-20%.... 10 = 91-100%). The evaluator was “blind” to the treatments and did not know what treatment was being rated. Discoloration was generally gray-brown or reddish-brown. Mid-point values of scale increments were used for data analysis.

2.3.5. Pre-inoculation high temperature in relation to root rot

A series of experiments was conducted to examine pre-inoculation episodes of high root-zone temperature in relation to root colonization by *P. aphanidermatum* and root discoloration (browning) after the roots were inoculated with the pathogen. For high temperature treatments the nutrient solution was maintained at 33°C for 0 (control), 1, 3, 6, 9, 12, 18, 24, 48, 72, 96, 120, 144, 168, 192, or 216 h. Roots were inoculated with *P. aphanidermatum*, or not inoculated, within an hour after the nutrient solution of the high temperature treatments had cooled to 23°C. For logistical reasons, effects of only four or five different periods of high temperature were investigated in any given experiment. The high temperature periods were overlapped among experiments to ascertain consistency of results and to provide a basis to combine data for presentation.
Controls included non-inoculated and inoculated plants maintained continuously at 23ºC, and non-inoculated plants exposed to the same high temperature periods as the inoculated plants. High temperature treatments within any given experiment were staggered so that all plants could be inoculated at the same time with the same batch of inoculum. There were three to six replicate plants per treatment and three or four repetitions of each experiment. Root colonization and discoloration were estimated daily.

2.3.6. Delayed inoculation as a factor affecting relationships of pre-inoculation high temperature and root rot

In a second series of experiments, time of inoculation following a high temperature episode in the root zone was examined as a possible variable influencing the effects of the episode on root colonization by *P. aphanidermatum* and root browning. In a standard high temperature treatment the nutrient solution was maintained at 33ºC for 72 h. At all other times the solution was kept at 23ºC except for the time required, respectively, to warm and cool the solution before and after the high temperature treatment. The nutrient solution of other plants was maintained continuously at 23ºC. Plants of each temperature treatment either were not inoculated or were inoculated with *P. aphanidermatum* at 0, 24, 48, 72, 96, 120, 144, and 216 h following the high temperature treatment. For logistical reasons, the effects of only four intervals between the high temperature treatment and inoculation were investigated in any given experiment. The interval treatments were overlapped among experiments to ascertain consistency among
results and to provide a basis to combine data for presentation. In each experiment, plants of each treatment were inoculated at the same time with the same batch of inoculum. In preliminary studies, it was found that the age differences of the plants when inoculated did not significantly affect progress of root browning. Root browning was estimated immediately before and after the high temperature treatment, immediately before the time of inoculation, and daily thereafter.

2.3.7. Experimental design and Statistical analysis

In all instances the experimental unit was a single plant in a hydroponic unit. Each experiment was designed as a randomized complete block and repeated three times. Trends in data of experimental repetitions were similar, and in each instance data of one repetition are presented. For each treatment water baths were used, each accommodating up to six hydroponic units. Great care was taken to avoid the extreme risk of cross contamination of non-inoculated hydroponic units with *P. aphanidermatum* during all experiments. There were three to five replicate plants per treatment in experiments. Each experiment was designed as a randomized complete block and conducted three times. Data from all three experimental repetitions could not be pooled because of slight differences between experiments, for example light intensity. The data were analyzed using the ANOVA procedure of SAS software Version 9.1 (SAS Institute Inc. Cary, N.C., USA). The ANCOVA procedure of SAS was used to
compare the slopes of the regression lines for hours at 33ºC versus root browning. The Type 1 error rate (\( \alpha \)) was set at 0.05.

2.4. Results

2.4.1. Pre-inoculation high temperature in relation to root rot

*Pythium aphanidermatum* was not recovered from roots of non-inoculated plants. Non-inoculated roots maintained continuously at 23ºC (0 h at 33ºC) appeared whitish and exhibited no discoloration throughout the experiment. Non-inoculated roots treated at 33ºC for up to 72 h also were symptomless but developed a pale straw color when treated for 96 to 216 h. The pathogen was recovered from all root segments of inoculated plants that were placed on the selective agar medium at 12 h following inoculation or later, including segments of roots that were not exposed to high temperature. Roots of inoculated plants maintained continuously at 23ºC (0 h at 33ºC) were symptomless until 4 days after the roots were inoculated with *P. aphanidermatum* when a few root tips appeared brown. The incidence of brown root tips subsequently increased to 100% during days 5 to 12 (Figure 2.2A). Expansive browning of the roots increased rapidly to 100% during days 6 to 12 after inoculation (Figure 2.2B). The curves for brown root tips and expansive root browning were similar in form and each increased in a linear pattern from approximately 5% severity to total browning. Disease increase in roots treated at 33ºC for 1, 3, or 6 h prior to inoculation with *P. aphanidermatum* did not differ significantly from increases observed in roots of the inoculated plants that were not treated at high temperature (i.e. 0 h at 33ºC, data not shown). This was true whether disease
Figure 2.2. Progress curves for percent brown root tips (A) and percent brown roots (B) in sweet peppers grown in single-plant hydroponic units in which the nutrient solution was 23°C and increased to 33°C for 0-216 h immediately before the roots were inoculated with *Pythium aphanidermatum*, or was not increased in control plants that were not inoculated. Symbols indicate treatments in terms of hours at 33 °C and inoculation or no inoculation with *P. aphanidermatum* as follows: ○ 0 h - *Pythium*; ▲ 0 h + *Pythium*; □ 1-216 h - *Pythium*; ■ 9 h + *Pythium*; ● 24 h + *Pythium*; ♦ 72 h + *Pythium*; X 144 h + *Pythium*. The experiment was repeated three times and results shown are from one representative replication. Data are the means ± SEM of 5 plants.
was assessed as brown root tips or general root browning. However, disease progressed approximately 1 day earlier in roots that were treated at 33°C for 9 h before inoculation compared those that were not exposed to 33°C (0 h) before inoculation (Figures 2.2A and 2.2B). The disease progress curves further show that root tip browning and expansive root browning developed progressively earlier as the duration of pre-inoculation high temperature was increased from 9 to 144 h. Curves for roots treated at 33°C for 12 and 18 h, 48 h, or 96 and 120 h, respectively (data not shown) fell between those for 9 and 24 h, 24 and 72 h, and 72 and 144 h. Curves for roots exposed to 33°C for various periods before inoculation were similar in shape and slope to those of inoculated plants maintained continuously at 23°C (0 h at 33°C), regardless of the duration of exposure to 33°C and of whether disease was expressed in terms of brown root tips or expansive root browning. Comparison of the slopes of the regression lines for values from 20% to 80% disease severity did not reveal significant differences among treatments (P = 0.05). The incidence of brown root tips and severity of expansive browning each reached 50% about 4.5 days earlier in plants treated at 33°C for 144 h compared to inoculated plants that were not exposed to 33°C (i.e. the 0 h treatment; Figures 2.3A, 2.3B). Precocious responses in terms of time to 50% disease were especially marked following 9 to 24 h of high root-zone temperature, whereas further increases up to 144 h exerted comparatively minor additional effects. Curves for root tip browning and expansive root browning following 168, 192, or 216 h of high root-zone temperature did not differ significantly from those that followed 144 h of high temperature.
Figure 2.3. Time (days) to 50% incidence of brown root tips (A) and to 50% root browning (B) in sweet peppers grown in single-plant hydroponic units in which the nutrient solution was 23°C and increased to 33°C for 0-216 h immediately before the roots were inoculated with *Pythium aphanidermatum*. The experiment was repeated three times the results for one repetition are shown. Data are the means ± SEM of 5 plants.
2.4.2. Delayed inoculation as a factor affecting relationships of pre-inoculation high temperature and root rot

No browning symptoms developed in non-inoculated plants with roots that were not exposed to 33°C (i.e. maintained continuously at 23°C) or in those exposed to the high temperature and assessed at 0, 72, 144, or 216 h later (data not shown). In inoculated plants not treated at 33°C the incidence of brown root tips increased linearly beginning at 4 days after inoculation and reached 50% and 100% at 8 and 11 days, respectively (Figure 2.4A). By comparison, root tip browning developed about 2 days earlier in plants that were inoculated immediately (0 h) following the high temperature treatment (33°C for 72 h), such that a few tips were brown at 2 days after inoculation, 50% were brown at 5.5 days, and 100% were brown at 9 days. In plants inoculated at 72 h after the high temperature treatment, root tip browning developed after approximately the same interval following inoculation and in a similar pattern to those found for plants inoculated immediately (0 h) following the high temperature episode. However, when inoculation was delayed until 144 h and 216 h after the high temperature treatment the progress curves for root tip browning were delayed approximately 1 and 2 days following inoculation, respectively, compared with plants inoculated immediately after the high temperature treatment, but nonetheless earlier than in inoculated plants that were not exposed to 33°C (i.e. maintained continuously at 33°C).

Expansive browning of roots of the inoculated plants that were not treated (i.e. maintained continuously at 23°C) increased almost linearly from 23°C 0 to
Figure 2.4. Progress curves for percent brown root tips (A) and percent brown roots (B) in sweet peppers that were grown in single-plant hydroponic units at 23°C and inoculated with *Pythium aphanidermatum* at 0-216 h after a standard episode of high nutrient solution temperature (33°C for 72 h), or were not treated at the high temperature and were then inoculated or not inoculated. Symbols indicate treatments in terms of high temperature treatment or not, hours at 23°C following treatment at 33°C and inoculation with *P. aphanidermatum* as follows: ○ no treatment, 0 h - *Pythium*; ▲ no treatment, 0 h + *Pythium*; □ no treatment, 1-216 h - *Pythium*; ● treatment, 0 h + *Pythium*; ▪ treatment, 72 h + *Pythium*; ◆ treatment, 144 h + *Pythium*; ■ treatment, 216 h + *Pythium*. The experiment was repeated three times and the results from one repetition are shown. Data are the means ± SEM of 4 plants.
100% between 4 and 11 days after inoculation 100% 100% between 4 and 11 days after inoculation (Figure 2.4B). Root browning developed about 2 days earlier, however in roots that were treated at high temperature and immediately inoculated. Progress of browning following high temperature treatment was progressively less precocious when root inoculation was delayed for 72, 144, and 216 h following the high temperature treatment (Figure 2.4B). In plants inoculated at 0, 72, 144, and 216 h after high temperature treatment, 50% of the roots were brown by 6, 7, 7, and 8 days after inoculation compared to 10 days for inoculated plants that were not treated at 33ºC.

2.5. Discussion

The observations demonstrate that a single period of high root-zone temperature (33ºC) of 9 h or longer predisposes vegetative pepper plants to precocious root tip browning and expansive root browning caused by *P. aphanidermatum*. Because the roots were exposed to high temperature only before inoculation, the observed precocious browning of the roots was mediated entirely through host responses to the high temperature episodes. All phases of disease development including root infection by the zoospores, biotrophic colonization of the roots by hyphae of the pathogen, and the necrotrophic phase when the roots turned brown (Sutton et al., 2006) developed while the temperature of the root zone was moderate (near 23ºC), thus precluding the possibility of any direct influence of high temperature on disease development.

While pre-inoculation high temperature evoked precocious browning of the
roots following inoculation with *P. aphanidermatum*, the rate of increase in brown roots was largely unaffected. As indicated by the slopes of the browning curves, the rate of browning was similar for all pre-inoculation high temperature treatments including the inoculated control plants that were not exposed to high temperature. In each instance browning symptoms increased from near zero to 100% within 7 to 8 days. The browning curves may have arisen entirely from the zoospores used to initiate disease in which case root rot development was monocyclic. Any zoospores produced in the root zones of hydroponic units during the brief period of disease increase would almost certainly have been immobilized, and thus inactivated, by the incessant turbulence produced by air bubbled into the nutrient solution to maintain dissolved oxygen levels (Sutton et al., 2006). Taken together, the data indicate that high pre-inoculation temperature of the root-zone shortens the biotrophic phase and thus advances the necrotrophic phase of root rot, but has little effect on the rate of expansive root browning once the necrotrophic phase is initiated.

The quantitative relationship between the duration of high pre-inoculation temperature and the time required following inoculation for the roots to turn brown exhibited three major attributes: a minimum duration threshold (approximately 6-9 h) below which no precocious response was detected, an intermediate range of 9-44 h in which increasing duration of high temperature evoked progressively earlier root browning, and a maximum duration threshold of 144-168 h which, if exceeded, did not further affect the time of root browning. Among the durations of pre-inoculation high temperature evaluated, periods of
144-216 h resulted in the most precocious and overlapping curves in which the roots turned brown approximately 4 days before those of inoculated control plants maintained continuously at 23°C. As indicated by the curves for times from inoculation to 50% root browning (Figure 2.3B), the precocious responses of root browning to incremental increases in duration of high temperature were more pronounced for periods of high temperature in the range of 6 to 24 h compared to 24 to 144 h. The shapes of the curves suggest that a saturation phenomenon was involved in the host response to lengthening episodes of high temperature. Possible contributions to precocious root browning of root-zone temperatures during periods of warming and cooling of the nutrient solution were not determined. A quantitative relationship between duration of pre-inoculation high temperature (35-45°C) and disease severity was also reported for black spruce needles inoculated with Botrytis cinerea (Zhang and Sutton, 1994).

The observed persistence of predisposition to root browning evoked by high root-zone temperature indicated that the predisposition to P. aphanidermatum was somehow “memorized” by the peppers. From the data of precocious root browning, predisposition was “memorized” for at least 216 h (9 days) following a high temperature episode of 72 h. More prolonged persistence was also possible given that the roots turned brown at least a day earlier in plants inoculated at 216 h compared to similar plants that were not exposed to high temperature. Intensity of pre-disposition was high for at least 72 h based on the similar precociousness of root browning when roots were inoculated at 0 or 72 h after high temperature treatment, and moderately high for 144 h based on the
substantially precocious browning curve obtained. The progressive decline in precociouslyness of browning when inoculation was delayed for more than 72 h suggested that the roots were slowly recovering from effects of high temperature treatment.

The observed predisposition to root browning was not attributed to oxygen depletion in the nutrient solution since the level of dissolved oxygen in the aerated nutrient solutions was maintained between 5 and 7 mg O₂ L⁻¹ solution and did not differ significantly when the solution temperature was 33°C compared with 23°C. However, the general decline in levels of dissolved oxygen with increasing temperature when nutrient solutions are not well aerated or oxygenated may enhance host predisposition to root rot, and so may be a factor in hydroponic pepper crops. Increased rates of development of root rot caused by *P. aphanidermatum* and other *Pythium* spp. when the temperature was high and concentration of dissolved oxygen was reduced could not be attributed entirely to host predisposition (Chérif et al., 2004). Many investigators reported greater severity of Pythium root rot when temperatures were high (e.g. 27-35°C) than when moderate (e.g. 20-25°C), but it was unclear whether the effects could be ascribed exclusively to high temperature or also to reduced oxygen levels, and whether the effects were mediated through the host or pathogen separately or in combination (Sutton et al., 2006). In the present study, host predisposition is attributed to high temperature but not to reduced oxygen levels in the nutrient solution during the pre-inoculation high temperature treatments.
Stress responses of the peppers to the high temperature treatments possibly played a role in the predisposition of the roots to browning. High temperature (heat) stress is a function of the rate of temperature increase and the intensity (in degrees) and duration of the stress-level temperature (Wahid et al., 2007). The findings that predisposition evoked by high temperature increased with treatment duration were consistent with reported plant responses to stress-level temperatures (Wahid et al., 2007) and with the notion that there is a relationship between stress responses and predisposition. The intensity of the high temperature treatments (33ºC) exceeded temperatures quoted as optimal or near-optimal for vegetative growth and fruit production in peppers (Andrews, 1984; Dodd et al., 2000) and so may have been sufficient to induce mild stress responses. Common responses of plants to moderate temperature stress include alterations in the homeostasis, stability, biosynthesis and compartmentalization of hormones and increased fluidity and permeability of cellular membranes (Wahid et al., 2007). When temperature is high, levels of the stress hormones abscisic acid (ABA) and ethylene generally increase, and production of cytokinins, which act to delay senescence, decrease (Taiz and Zeiger 2006, Wahid et al., 2007). In response to stress, ABA promotes senescence and stimulates the production of ethylene, which regulates numerous growth and developmental processes in plants and accelerates tissue senescence (Taiz and Zeiger, 2006; Wahid et al., 2007). In a study with explants of pepper (Capsicum annuum), ethylene production was greater at 34ºC than at 25, 42 and 48ºC (Aloni et al., 1994). Huberman et al. (1997) reported a positive correlation between high temperature
and the level of 1-amino-cyclopropane-1-carboxylic acid, a precursor of ethylene biosynthesis, in the pepper genus *Piper nigrum*. Taken together, the physiological responses of roots to high temperature stress are similar to physiological changes that occur during senescence (Taiz and Zeiger, 2006). It is plausible that physiological changes in the roots following high temperature treatment such as increased permeability of the cell membranes, enhanced leakage of ions and energy sources into the intercellular spaces, and reduced tissue resistance to the pathogen may have favored intensified root colonization by *P. aphanidermatum* during the biotrophic phase. In agreement with findings of Owen-Going et al. (2008), the pathogen was recovered from all portions of root systems within 12 h after the roots were inoculated, however densities of hyphae in the root tissues were not determined. To explain the precocious browning in predisposed roots, it is hypothesized that root browning develops as a function of colonization intensity of the roots by *P. aphanidermatum*.

Browning of pepper roots infected by *P. aphanidermatum* is associated with phenolic polymers that accumulate in the tissues and bind to the cell walls (Owen-Going et al., 2008; Sutton et al., 2003). Phenolic compounds in higher plants are derived at least in part from phenylalanine, a product of the Shikimic acid pathway (Taiz and Zeiger, 2006). Deamination of phenylalanine by phenylalanine ammonia lyase (PAL) yields *trans*-cinnamic acid and its phenylpropanoid derivatives which are important building blocks for more complex phenolic compounds. Taken together, available evidence indicates that *P. aphanidermatum* activates the Shikimic acid and phenylpropanoid pathways.
and promotes biosynthesis of phenolic compounds in infected roots (Owen-Going, 2006). Elicitors of *P. aphanidermatum* and other pathogens were reported to increase PAL in cultured plant cells and protoplasts (Schnitzler and Seitz 1989). A possible activator of the phenylpropanoid pathway, and thus a trigger for root browning, is the metabolite referred to as the *P. aphanidermatum* necrosis-inducing elicitor, or PaNie (Veit et al., 2001). Conditions that initiate or stimulate production of elicitors or other factors that trigger root browning, and thus the end of the biotrophic phase, are not understood. It is suggested that intensified colonization of roots by *P. aphanidermatum* following high temperature predisposition mediates early synthesis of PaNie to thresholds sufficient to trigger precocious browning of the roots. The findings that pre-inoculation high temperature had little effect on the rate of root browning under constant moderate temperature (23°C), and that browning increased continuously to about 100% in all treatments, may indicate that browning becomes autocatalytic once triggered.

The quantitative observations of high root-zone temperature in relation to root browning contribute further understanding of Pythium root rot epidemiology and have implications for rational management of the disease in commercial pepper crops. Based on my data, populations of predisposed but otherwise healthy peppers in hydroponic greenhouses are at risk of precocious and rapid root rot development should they become infected by *P. aphanidermatum*. The persistence of predisposition over at least 9 days has implications for pepper health management. For example, peppers exposed to high temperature as
transplants could remain predisposed to root rot after they are transferred to production greenhouses. In another possible scenario, shortening of infection cycles of *P. aphanidermatum* on account of host predisposition may favor zoospore production and accelerate progress of root rot in commercial pepper crops, even during early stages of epidemics when rates of increase in root browning (i.e. slopes of browning curves) are comparatively low (Owen-Going, 2002). The observations of high temperature and pre-disposition provide a rational basis for estimating the risk of precocious disease increase and the available window of opportunity for taking preventative or re-medial measures such as through the use of beneficial microbes (Corrêa et al., 2009). Prediction of precocious disease could be further refined based on additional data, such as of serial episodes of high temperature which might act cumulatively, additively or otherwise in predisposing pepper plants to root rot. However the finding that a single episode of 6 to 9 h at 33°C is sufficient to predispose peppers to Pythium root rot underscores the importance of minimizing high temperature episodes for managing root health and promoting productivity of hydroponic pepper crops.
Chapter 3. Quantitative relationships of *Pseudomonas chlororaphis* 63-28 to Pythium root rot and growth in hydroponic peppers

3.1. Abstract

The ability of *Pseudomonas chlororaphis* 63-28 to suppress Pythium root rot (*Pythium aphanidermatum*) and promote plant growth was investigated in hydroponic peppers that were predisposed or not predisposed to the disease. The biocontrol agent was introduced into the nutrient solution 10 days before the roots were inoculated with the pathogen. The root zone was maintained at 23°C except when roots were exposed to 33°C for 3 days before inoculation to induce predisposition to root rot. At constant 23°C (no predisposition) application of *P. chlororaphis* at $10^7$ CFU mL$^{-1}$ nutrient solution delayed root browning more effectively than did higher or lower densities. In predisposed plants, densities of $10^6$, $10^7$ and $10^8$ CFU mL$^{-1}$ were equally superior at delaying root browning than $10^4$ and $10^5$ CFU mL$^{-1}$. When applied at $10^7$ CFU mL$^{-1}$, the density of *P. chlororaphis* on roots of the two temperature regimes ranged from log$_{10}$ 5.88 to 6.45 CFU g$^{-1}$ fresh roots at 7 to 19 days after application. The agent delayed root browning, re-mediated predisposition to root rot, and increased growth of inoculated and non-inoculated plants. Leaf expansion was a sensitive marker of root rot and remediation by *P. chlororaphis*. I conclude that *P. chlororaphis* 63-28 has substantial potential for managing the disease regardless of predisposition.
3.2. Introduction

Sweet peppers (*Capsicum annuum* L.) are produced year round in Ontario greenhouses by means of sophisticated hydroponic systems. The pepper plants normally are grown in slabs of rockwool or coconut fibre, or in troughs, through which plant nutrient solution is continuously re-circulated. The solution is discharged from the root zone into mixing tanks, in which the nutrient composition, pH, and oxygen level are regulated, and subsequently returned to the root zone. Heavy and sustained productivity of marketable fruit during the crop cycle is paramount for pepper crops to be competitive and profitable, but is frequently compromised by the development of Pythium root rot. In Ontario, root rot is caused primarily by *Pythium aphanidermatum* (Edson) Fitzp. and is characterized by browning (necrosis) and fragmentation of the roots (Owen-Going et al., 2003; Sutton et al., 2006). Severe root rot often develops in commercial crops during or following periods of warm or sunny weather when the root-zone temperature is high (e.g. 28 to 34°C) (Sutton et al., 2006). Research performed in Chapter two of this thesis confirms that high root-zone temperature favours rapid development of root rot and that roots of healthy peppers are predisposed to early and severe root browning when exposed to 33°C for nine to 144 h and subsequently inoculated with *P. aphanidermatum*.

Colonization of pepper roots by *P. aphanidermatum* is normally biotrophic at first and subsequently necrotrophic (Sutton et al., 2006). The biotrophic phase, during which the roots generally remain whitish and symptomless, is frequently prolonged (e.g. several weeks) and sometimes indefinite when the root zone is
cool (e.g. 16-20°C), but the transition to necrotrophy (root browning) occurs within days or even hours at higher temperatures (e.g. 24 to 34°C) (Sutton et al., 2006). Growth and productivity of peppers and other plants are usually reduced after the roots turn brown, but not in the biotrophic phase. Ortiz-Uribe (2007) found that root and shoot growth of hydroponically-grown snapdragons abruptly and markedly declined at the onset of the necrotrophic phase of Pythium root rot. Thus, available information indicates that control of root browning is critical for maintaining high productivity in *P. aphanidermatum*-infected crops, but effective and practical methods to achieve this are lacking.

Root-zone temperatures that are sufficiently cool to control root browning are sub-optimal for pepper growth and in many instances difficult and prohibitively expensive to maintain. Beneficial microbes are potential means for controlling root rot, but have received only limited attention in hydroponic peppers (Corrêa and Bettiol, 2009; Corrêa et al., 2012). Several workers (e.g. McCullagh et al., 1996; Sutton et al., 2008) concluded that certain microbial agents have value for enhancing the growth and productivity of healthy as well as diseased crops and justify development as plant inoculants. Microbial treatments also have the potential to satisfy safety requirements for greenhouse employees, consumers, and the environment.

Microbes were reported to suppress Pythium root rot caused by *P. aphanidermatum* in several kinds of hydroponic crops, notably cucumbers, chrysanthemums, tomatoes, and lettuce (Corrêa and Bettiol, 2009; Corrêa et al., 2010). Those with superior effectiveness in lab tests included strains of *Bacillus*
subtilis, Bacillus cereus, Streptomyces griseoviridis, Pseudomonas fluorescens, Pseudomonas corrugata, Pseudomonas chlororaphis, Comamonas acidovorans, Clonostachys rosea, and Trichoderma harzianum (Rankin and Paulitz, 1994; McCullagh et al., 1996; Paulitz and Bélanger, 2001; Zheng et al., 2000; Liu et al., 2003; Liu et al., 2007). Strains of Pseudomonas (P.) chlororaphis (= Pseudomonas aureofaciens) (Johnson and Palleroni, 1989) strongly suppressed P. aphanidermatum in roots of pepper, cucumber, chrysanthemum and snapdragon in small-scale hydroponic systems and P. chlororaphis generally performed better than isolates of other microbes (Chatterton, 2002; Chatterton et al., 2004; Khan et al., 2003; Liu et al., 2007). Pseudomonas chlororaphis strains TX-1 and 63-28 (Turf Science Laboratories, National City, CA, USA) were of similar effectiveness in suppressing root rot caused by P. aphanidermatum in chrysanthemums grown in single-plant hydroponic units in a greenhouse at 22-25°C, and also performed well against the pathogen when the root-zone temperature was high (32°C) (Liu et al., 2007). Liu et al. (2007) further found that P. chlororaphis strain 63-28, but not strain TX-1, was effective against chrysanthemum root rot caused by Pythium dissotocum, which was reported as a pathogen on hydroponic peppers in Ontario (Owen-Going et al., 2003). Pseudomonas chlororaphis 63-28 suppressed Pythium root rot and promoted shoot growth more effectively than did other agents in vegetative-stage cucumbers grown in greenhouse hydroponic systems (Liu et al., 2003). McCullagh et al. (1996) found that the number of fruit produced over time in cucumbers inoculated with P. aphanidermatum was greater in plants treated with
*P. fluorescens* 63-28 (= *P. chlororaphis* 63-28) compared to untreated plants, but found no significant effect on yield when disease severity was low or absent. Gagné et al. (1993) reported that *P. fluorescens* 63-28 substantially increased the total fruit yield and percentage of marketable fruit of tomatoes grown in peat-based media amended with the bacterium prior to planting compared to plants grown in non-amended media. Taken together, the reports suggest that *P. chlororaphis* 63-28 justifies further investigation for dual activity as an inoculant to enhance productivity of pepper crops in which little or no disease is present and as an agent to suppress Pythium root rot. Findings in Chapter two of this thesis that high root zone temperature predisposes pepper plants to Pythium root rot underscore the need to examine effectiveness of treatments in predisposed as well as non-predisposed plants.

The objectives of the present investigations in hydroponically-grown peppers were: 1, to determine relationships of inoculum density of *P. chlororaphis* 63-28 applied in the nutrient solution to progress of root browning caused by *P. aphanidermatum*; 2, to determine the optimum density of *P. chlororaphis* 63-28 inoculum for root rot control; 3, to examine densities of *P. chlororaphis* 63-28 associated with the roots as a function of time after application, root zone temperature regimes, and inoculation with *P. aphanidermatum*; 4, to examine quantitative effects and interactions of *P. chlororaphis* 63-28, root-zone temperature regimes, and *P. aphanidermatum* on growth parameters of vegetative peppers; and 5, to assess leaf expansion as a
minimally intrusive means to estimate effectiveness of *P. chlororaphis* 63-28 against root rot.

3.3. Materials and Methods

3.3.1. Hydroponic pepper plants

Plants of sweet pepper cv. Cubico (DeRuiter Seeds Inc., Columbus, OH, USA) were grown in a growth room as described in Chapter two of this thesis. In brief, 5-week old seedlings in rockwool plugs (2.5 cm X 2.5 cm X 4.0 cm; Grodan, Roermond, The Netherlands) were placed in 5-cm diameter mesh pots and transferred to single-plant hydroponic units. Each unit comprised of a 475 mL white polyethylene container with a lid in which a hole was made to accommodate the mesh pot and plant. The container was filled with a plant nutrient solution that was aerated continuously by means of a bubbler. When used for experiments, plants were initially 9-11 cm tall with roots that extended 8-10 cm below the rockwool plugs into the nutrient solution. The air temperature in the growth room was 23-24°C and the intensity of photosynthetically active radiation (PAR) at plant height was 180-200 µmol/m²/s as measured by quantum sensors (Q 3991-4 LI-COR Inc., Lincoln, NE, USA).

3.3.2. Control of high root-zone temperature

The hydroponic units were placed in temperature-controlled water baths to control root-zone temperature as described in chapter two. The baths were positioned on the bench in the growth room.
3.3.3. Inoculum production and application of *Pseudomonas chlororaphis*

*Pseudomonas chlororaphis* strain 63-28 was used in experiments. For long-term storage, cells were suspended in glycerol:tryptic soy broth (TSB) (30:70) and kept in 1 mL microfuge tubes at -20°C and -70°C. The bacterium was recovered on tryptic soy agar medium (TSA) at 22°C for 48 h and subsequently grown in TSB in Erlenmeyer flasks on a gyratory shaker (Model G2, New Brunswick Scientific, Edison, NJ, USA) operated at 100 rpm. For tests on pepper plants, 24-h TSB cultures were centrifuged under refrigeration at 3000 x g for 15 min. Recovered cells were washed twice and re-suspended in 0.1 M MgSO₄. The density of suspended cells was estimated from absorbance values obtained at 600 nm on a spectrophotometer (model PU8620, Philips, Cambridge, UK) and a standard curve of absorbance vs. cell density. Cells were centrifuged again, suspended in nutrient solution at a density of 1 X 10⁹ colony forming units (CFU) mL⁻¹ and applied into the nutrient solution of the hydroponic units 10 days before the roots were inoculated with *P. aphanidermatum*. The final density of *P. chlororaphis* in the nutrient solution of the units was 1 X 10⁷ CFU mL⁻¹ unless otherwise indicated.

3.3.4. *Pythium* inoculum and inoculations

The protocols used for maintenance and growth of *P. aphanidermatum* and for inoculating pepper roots were described in Chapter two of this thesis. In summary the pathogen was maintained on root tissue, re-isolated on a *Pythium*-selective agar medium, and induced to sporulate in Petri dishes containing V8
agar medium. For inoculations, each plant was lifted from its hydroponic unit, positioned in a metal tray with all roots immersed in an aqueous suspension of $5 \times 10^3$ zoospores mL$^{-1}$ for 30 min, and returned to the unit.

3.3.5. Density of *Pseudomonas chlororaphis* in roots

Numbers of *P. chlororaphis* cells recovered from the roots were estimated by grinding composite 1-g root samples in 10 mL aliquots of 0.1 M MgSO$_4$ using sterilized mortars and pestles. The samples included randomly-collected roots of all orders and depths in the hydroponic units. Suspensions of the ground tissues were serially diluted, spread onto TSA medium with five replications in Petri dishes, and incubated at 25°C for 48-72 h. There were five replications plated for each dilution. Colony counts were used to estimate CFU g$^{-1}$ fresh roots.

3.3.6. Root colonization by *Pythium aphanidermatum* and root discoloration

To estimate percent roots colonized by *P. aphanidermatum*, roots were detached at random from each plant and cut into 1-cm long segments. The segments were surface disinfested in 1% NaOCl for 30 s and in 70% ethanol for 30 s, rinsed three times in sterile distilled water and blotted dry. Thirty random root segments from each plant were placed on the *Pythium*-selective medium, incubated at 25°C for 48 h, and assessed for incidence of *P. aphanidermatum*.

For estimating root discoloration, each plant was lifted above the nutrient solution and the roots were examined with the aid of a hand lens (10X magnification). The percent of roots that were discolored was estimated using an
equal-increment scale of 0-10 (0 = 0%, 1 = 1-10%, 2 = 11-20%.... 10 = 91-100%). The evaluator was “blind” to the treatments and did not know what treatment was being rated. Discoloration was usually gray-brown or reddish-brown. Mid-point values of scale increments were used for data analysis.

3.3.7. Plant growth analysis

In destructive harvests, the height of each plant was measured from the rockwool plug to the highest point of the apical cluster of leaves, and the shoots were removed and immediately weighed to determine fresh mass. All leaves were detached and a leaf area meter (model LI-3000, LI-COR, Lincoln, NE, USA) was used to measure total leaf area per plant. All roots exterior to the rockwool cube were removed, blotted dry and weighed to determine fresh mass. To determine dry mass values, the shoots (stems plus leaves) and roots were dried to constant weight at 80°C for 48 h in a drying oven and weighed.

3.3.8. Experiment 1: Progress of Pythium root rot in relation to density of Pseudomonas chlororaphis

Pseudomonas chlororaphis was applied into the nutrient solution of the hydroponic units at final densities of 0, $10^4$, $10^5$, $10^6$, $10^7$, and $10^8$ CFU mL$^{-1}$. After seven days at 23°C, the temperature of the nutrient solution of half of the treated plants and half of the untreated plants was raised to 33°C for three days to predispose the plants to root rot and subsequently cooled to 23°C. The solution of the remaining plants was constant at 23°C (no predisposition). Plants
of each *P. chlororaphis* treatment in each temperature regime were inoculated or not inoculated with *P. aphanidermatum* 10 days after *P. chlororaphis* was applied. The nutrient solution of high temperature treatments was cooled to 23˚C before roots were inoculated. Root discoloration was estimated immediately before the plants were treated with *P. chlororaphis*, immediately before the pathogen was introduced into the root zone, and each day thereafter for 12 days.

3.3.9. Experiment 2: Density dynamics of *Pseudomonas chlororaphis* in the roots as a function of time after application in the root zone, temperature of the root zone and root inoculation with *Pythium aphanidermatum*

The nutrient solution of hydroponic pepper plants was treated with *P. chlororaphis* at a final density of 1 X 10⁷ CFU/mL or not treated. Root zone temperatures, pathogen inoculations, and the method used to estimate root discoloration were the same as in experiment 1. Root colonization by *P. aphanidermatum* was estimated at 0, 3, 6, 9, 12, 15, 24, 36 and 48 h after inoculation. Density of *P. chlororaphis* associated with the roots was estimated immediately before and after the time of the high temperature predisposition treatment, and at 3, 6, and 9 days after roots were inoculated with the pathogen (respectively 7, 10, 13, 16, and 19 days after *P. chlororaphis* was applied into the nutrient solution).
3.3.10. Experiment 3. Effects of *Pseudomonas chlororaphis*, root-zone temperature, and root inoculation with *Pythium aphanidermatum* on plant growth parameters

Plants were treated with *P. chlororaphis* 63-28, exposed to high temperature and inoculated with *P. aphanidermatum* as described for experiment 2. Plant growth analyses (described above) were performed immediately before *P. chlororaphis* 63-28 was applied in the nutrient solution, immediately before roots were inoculated with *P. aphanidermatum*, and at 3, 6, 9, and 12 days after inoculation.

3.3.11. Experiment 4: Effects of *Pseudomonas chlororaphis*, root-zone temperature, and root inoculation with *Pythium aphanidermatum* on leaf expansion

Plants were treated with *P. chlororaphis*, exposed to high temperature and inoculated with *P. aphanidermatum* as described above. A young attached leaf just below the shoot apex of each plant (leaf 12 from the base of the stem) was tagged immediately before *P. chlororaphis* was applied in the nutrient solution, and its area was measured immediately and daily thereafter. For area measurements, the leaf margin was traced onto a transparent photocopy sheet and the traced outline was excised and weighed. Leaf area was estimated from a linear standard curve for sheet area in relation to weight ($r^2 = 0.9997$). In a preliminary study conducted under the same conditions no significant difference
(P = 0.05) was found between the ratio of leaf area to leaf weight of healthy plants compared to plants with 60-80% root rot.

3.3.12. Experimental design and statistical analysis

In all instances the experimental unit was a single plant in a hydroponic unit. Each experiment was designed as a randomized complete block and repeated three times, except for experiment 4 which was repeated twice. Trends in data of experimental repetitions were similar, and in each instance data of one repetition are presented. Data from different experimental repetitions could not be pooled because of slight differences between experiments, for example light intensity. There were four replicate plants per treatment in experiments 1, 3 and 4 and three in experiment 2. Data were analysed with SAS software Version 9.1 (SAS Institute Inc. Cary, N.C., USA) using the ANOVA and LSD (P = 0.05) procedures for separation of treatment means. Regression lines for density of P. chlororaphis versus root browning were compared using PROC GLM ANCOVA in SAS.

3.4. Results
3.4.1. Experiment 1: Progress of Pythium root rot in relation to density of Pseudomonas chlororaphis

Roots of the two temperature treatments that were treated or not treated with P. chlororaphis and not inoculated with P. aphanidermatum appeared whitish and remained free from P. aphanidermatum throughout the experiment.
Roots not treated with *P. chlororaphis*, but inoculated with the pathogen turned brown approximately three days earlier when exposed to 33°C for three days prior to inoculation (i.e. roots predisposed to root rot) compared to those kept at a constant 23°C (not predisposed) (Figure 3.1). After onset, root browning in both instances progressed from 0 to 100% during seven to eight days. Application of *P. chlororaphis* at $10^4$ CFU mL$^{-1}$ nutrient solution had little or no effect on root browning, but application at $10^5$ CFU mL$^{-1}$ delayed browning by about three days in plants of each temperature treatment. When applied at respective final densities of $10^6$, $10^7$ and $10^8$ CFU mL$^{-1}$, *P. chlororaphis* delayed initial browning by four, four, and three days in roots at a constant 23°C and in each instance by six days in roots exposed to 33°C prior to inoculation with *P. aphanidermatum*. Slopes of the root browning curves that reached 95-100% during the experiment, calculated based on the initial five values exceeding zero, did not differ significantly within a repetition and trends between repetitions were similar (Table 3.1).

3.4.2. Experiment 2: Density dynamics of *Pseudomonas chlororaphis* in the roots as a function of time after application in the root zone, root-zone temperature and root inoculation with *Pythium aphanidermatum*

Roots of the two temperature regimes that were treated or not treated with *P. chlororaphis* and not inoculated with *P. aphanidermatum* appeared whitish throughout the experiment. The pathogen was not recovered from non-inoculated roots. Incidence of recovery of *P. aphanidermatum* from segments of inoculated roots was 0% for samples taken at 0 or 3 h after inoculation, respectively, 12%,
Figure 3.1. Root browning progress curves in hydroponic pepper plants inoculated with *Pythium aphanidermatum* (*Pa*) as a function of density of *Pseudomonas chlororaphis* 63-28 (*Pc*) applied in the nutrient solution and high temperature predisposition of the plants to Pythium root rot. *Pc* was applied 10 days before the roots were inoculated with *Pa*. The root zone was maintained at 23 °C for non-predisposed plants or at 33°C for three days prior to inoculation to predispose plants to root rot. The experiment was repeated three times and results from the first replication are shown. Data are the means ± SEM of 4 plants.
Table 3.1. Equations of the lines for root browning progress of hydroponic peppers that were treated or not treated with *Pseudomonas chlororaphis (Pc)*, predisposed or not predisposed to high temperature treatment and inoculated with *Pythium aphanidermatum*.

<table>
<thead>
<tr>
<th>Log$_{10} P_c$ density</th>
<th>Plants predisposed</th>
<th>Repetition 1</th>
<th>Repetition 2</th>
<th>Repetition 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated -</td>
<td>y=17.5x-94.3</td>
<td>y=18.2x-112.8</td>
<td>y=14.0x-86.0</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>y=17.5x-108.1</td>
<td>y=16.7x-101.2</td>
<td>y=13.8x-93.4</td>
<td></td>
</tr>
<tr>
<td>Untreated +</td>
<td>y=18.1x-43.7</td>
<td>y=16.9x-50.3</td>
<td>y=14.3x-45.2</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>y=17.1x-32.3</td>
<td>y=16.8x-57.6</td>
<td>y=13.7x-40.7</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>y=16.7x-30.9</td>
<td>y=15.9x-68.5</td>
<td>y=13.7x-43.8</td>
<td></td>
</tr>
</tbody>
</table>

The experiment was repeated three times, with 4 plants per treatment. Linear regressions of data for values from 20% to 80% disease severity were used to generate the equations of the lines. Results shown in Figure 3.1 are from the first repetition. Equations do not differ significantly within a repetition according to an ANCOVA test (P > 0.05).
46% and 70% for samples taken at 6, 9 and 12 hours, and 100% for subsequent samples. Pathogen incidence did not differ significantly \((P = 0.05)\) for roots that were treated or not treated with \(P.\ chlororaphis\), or for roots of the two temperature regimes, and root inoculation with \(Pythium\ aphanidermatum\). A mean density of log 6.42 CFU of \(P.\ chlororaphis\) g\(^{-1}\) roots was found seven days after the agent was applied into the nutrient solution (i.e. when high temperature predisposition treatments were initiated) (Figure 3.2). At 10 days after application (i.e. immediately before roots were inoculated with \(P.\ aphanidermatum\)) density of \(P.\ chlororaphis\) ranged from log 6.38 to 6.45 CFU g\(^{-1}\) roots. The high temperature predisposition treatment did not significantly affect density of \(P.\ chlororaphis\) at any time during the experiment except for a minor effect in inoculated plants at day 6. At 3, 6 and 9 days after inoculation with \(P.\ aphanidermatum\) (i.e. 13, 16, and 19 days after \(P.\ chlororaphis\) was applied into the nutrient solution) density of \(P.\ chlororaphis\) in inoculated roots was significantly higher than in non-inoculated roots. Agent densities declined significantly \((P = 0.05)\) between days six and nine in inoculated and non-inoculated plants. At 12 days, roots not treated with \(P.\ chlororaphis\) but inoculated with \(P.\ aphanidermatum\) were substantially disintegrated and meaningful estimates of CFUs could not be obtained.

The root browning curves did not differ significantly from those of the same treatments and conditions in Experiment 1. \(Pseudomonas\ chlororaphis\) delayed initial browning of inoculated roots by 7 days for plants that were exposed to 33°C compared to 5 days for plants at a constant 23°C. In each instance, browning did
Figure 3.2. Estimated density of *Pseudomonas chlororaphis* 63-28 (*Pc*) in hydroponic pepper roots as a function of time after application, high temperature predisposition of the plants to root rot, and root inoculation with *Pythium aphanidermatum* (*Pa*). *Pc* was applied in the nutrient solution at a final density of 1 X 10^7 CFU mL^-1 10 days before roots were inoculated with *Pa*. The root zone was maintained at 23ºC for non-predisposed plants, but the temperature was increased to 33ºC for three days prior to inoculation to predispose plants to root rot. Symbols indicate treatments in terms of high temperature treatment or not, and inoculation or no inoculation with *P. aphanidermatum* as follows: ○ no treatment, - *Pythium*; ● no treatment, + *Pythium*; □ treatment, - *Pythium*; ■ treatment, + *Pythium*. The experiment was repeated three times and results from one repetition are shown. Data are the means ± SEM of 5 dilution plates.
not exceed 30% when the experiment ended at 12 days after inoculation (22 days after \textit{P. chlororaphis} was applied.

3.4.3. Experiment 3. Effects of \textit{Pseudomonas chlororaphis}, root-zone temperature, and root inoculation with \textit{Pythium aphanidermatum} on plant growth parameters

\textit{Pseudomonas chlororaphis} did not significantly affect plant height, or fresh and dry mass values of the shoots and roots at seven days after application when the high temperature predisposition treatment was initiated, at 10 days when roots were inoculated with \textit{P. aphanidermatum}, or at 13 days (three days after inoculation), but significant effects were found at 16, 19, and 22 days (6, 9, and 12 days after inoculation). Significant increases in total plant leaf area were first observed at 10 days after the \textit{P. chlororaphis} treatment (the day of inoculation). At 19 days (9 days after inoculation), the shoots and root systems of plants treated with \textit{P. chlororaphis} generally appeared larger than those of untreated plants, regardless of the root zone temperature regime or whether the roots were inoculated with \textit{P. aphanidermatum} (Figure 3.3). Root rot was markedly less severe in \textit{P. chlororaphis}-treated plants than in untreated plants in each temperature regime.

3.4.3.1. Plant height

Plants that were not treated with \textit{P. chlororaphis} but inoculated with \textit{P. aphanidermatum} were significantly shorter than plants of all other treatments at 6
Figure 3.3. Pictorial comparison of the effects of *Pseudomonas chlororaphis* 63-28 (*Pc*) and inoculation of the roots with *Pythium aphanidermatum* (*Pa*), separately and in combination, in pepper plants that were not predisposed or predisposed to Pythium root rot. Plants were photographed at 9 days after inoculation with *Pa*. *Pc* was applied in the nutrient solution 10 days before the roots were inoculated with *Pa*. The root zone of non-predisposed plants was maintained at 23°C or at 33°C for three days for predisposed plants prior to inoculation with *Pa*. The experiment was repeated three times and the plants in the photo are representative of each treatment.
Table 3.2. Growth components of hydroponic peppers that were treated or not treated with *Pseudomonas chlororaphis* (*Pc*), predisposed or not predisposed to high temperature treatment, and inoculated or not inoculated with *Pythium aphanidermatum* (*Pa*). Data are presented for day 9 after inoculation with *P. aphanidermatum* (i.e. 19 days after application of *Pc*).

<table>
<thead>
<tr>
<th><em>Pc</em> Plants predisposed</th>
<th><em>Pa</em> Plant height (cm)</th>
<th>Total leaf area (cm$^2$)</th>
<th>Shoot fresh mass (g)</th>
<th>Shoot dry mass (g)</th>
<th>Root fresh mass (g)</th>
<th>Root dry mass (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>-</td>
<td>-</td>
<td>26.0c</td>
<td>1404c</td>
<td>62.9c</td>
<td>5.4c</td>
<td>11.8b</td>
</tr>
<tr>
<td>+</td>
<td>-</td>
<td>31.0a</td>
<td>1727a</td>
<td>70.6a</td>
<td>6.3a</td>
<td>13.8a</td>
</tr>
<tr>
<td>-</td>
<td>+</td>
<td>23.3d</td>
<td>1114d</td>
<td>54.1d</td>
<td>4.8d</td>
<td>10.1c</td>
</tr>
<tr>
<td>+</td>
<td>-</td>
<td>28.3b</td>
<td>1621ab</td>
<td>61.6c</td>
<td>5.4c</td>
<td>13.0ab</td>
</tr>
<tr>
<td>-</td>
<td>+</td>
<td>25.7c</td>
<td>1439c</td>
<td>60.4c</td>
<td>5.3c</td>
<td>11.7b</td>
</tr>
<tr>
<td>+</td>
<td>-</td>
<td>30.7a</td>
<td>1695a</td>
<td>72.6a</td>
<td>6.4a</td>
<td>13.6a</td>
</tr>
<tr>
<td>-</td>
<td>+</td>
<td>23.7d</td>
<td>1075d</td>
<td>51.3d</td>
<td>4.8d</td>
<td>9.1c</td>
</tr>
<tr>
<td>+</td>
<td>+</td>
<td>28.7b</td>
<td>1513bc</td>
<td>62.2bc</td>
<td>5.6bc</td>
<td>12.6b</td>
</tr>
</tbody>
</table>

The experiment was repeated three times and results for one repetition are shown. Data are the means ± SEM of 4 plants. Treatment means in a column followed by the same letter do not differ significantly (P > 0.05) according to ANOVA and LSD tests.
and 9 days after inoculation (16 and 19 days after \textit{P. chlororaphis} treatment) but not at earlier sampling times. At 9 days after inoculation, plant height in given treatments of the two temperature regimes did not differ significantly (Table 3.2). Plants that were untreated and not inoculated with \textit{P. aphanidermatum} averaged 26 cm in height. The pathogen suppressed the height of untreated plants by 8-10%. The \textit{P. chlororaphis} treatment increased plant height by 18-19% in plants not inoculated with the pathogen and by 10-11% in the inoculated plants.

3.4.3.2. Total plant leaf area
The mean total leaf area per plant was 270 cm\(^2\) when \textit{P. chlororaphis} was applied in the nutrient solution. After 7 days, when the high temperature treatment was initiated, the respective mean leaf areas of \textit{P. chlororaphis}-treated plants and untreated plants were 500 cm\(^2\) and 430 cm\(^2\) and not significantly different. After 10 days, when plants were inoculated with \textit{P. aphanidermatum}, leaf area was significantly greater in \textit{P. chlororaphis}-treated plants (815-840 cm\(^2\)) than in untreated plants (710-730 cm\(^2\)). The root-zone temperatures did not significantly affect leaf area of plants of given treatments at 10 days or at later sampling times. At 19 days (9 days after inoculation) in plants not treated with \textit{P. chlororaphis}, leaf area was 21-25% lower in inoculated compared to non-inoculated plants (Table 3.2). By 19 days following application, \textit{P. chlororaphis} increased leaf area by 18-23% in plants not inoculated with the pathogen and by 45-58% in the inoculated plants.
3.4.3.3. Fresh mass and dry mass

At 10 days after the *P. chlororaphis* treatment, when plants were inoculated with *P. aphanidermatum*, values for shoot fresh mass, shoot dry mass, root fresh mass and root dry mass, respectively, ranged from 39.05-42.80 g, 3.53-3.66 g, 6.35-7.07 g and 0.55-0.61 g and did not differ significantly between the two temperature regimes (data not shown). Values of the respective variables also were not significantly different on day 13 (3 days after inoculation). At day 16 (6 days after inoculation), no significant effects were found of *P. aphanidermatum* on fresh mass or dry mass values of shoots or roots of plants not treated with *P. chlororaphis* in either temperature regime, and values for the two regimes did not differ significantly. At that time in non-inoculated plants, *P. chlororaphis* significantly increased shoot fresh mass and dry mass by 4% and 5% respectively when the root zone was constant at 23°C, and by 10% and 12% in the high temperature regime. In the inoculated plants, values of the respective variables and temperature conditions were increased by 7% and 9% and by 7% and 10%. In the high temperature regime, the *P. chlororaphis* treatment increased root fresh mass and dry mass by 14% and 11%, respectively, in plants inoculated with *P. aphanidermatum* and by 12% and 10% in non-inoculated plants, but no treatment effects were observed in plants kept at a constant 23°C.

Major effects of the *P. chlororaphis* treatment and *P. aphanidermatum* on fresh mass and dry mass of the shoots and roots were observed at 19 days after the treatment (nine days after inoculation) (Table 3.2). The values for plants of the high temperature predisposition treatment were not significantly different from
those at a constant 23°C. In the absence of *P. chlororaphis*, *P. aphanidermatum* respectively suppressed shoot fresh mass and dry mass by 14-15% and 9-11%, and root fresh mass and dry mass by 14-22% and 16-22%, respectively. In plants treated with *P. chlororaphis* but not inoculated with *P. aphanidermatum*, shoot fresh mass was increased by 12-20%, shoot dry mass by 17-21%, root fresh mass by 16-17% and root dry mass by 20% compared to the untreated controls. In plants treated with *P. chlororaphis* and inoculated with *P. aphanidermatum*, shoot fresh mass was increased by 14-21%, shoot dry mass by 13-17%, root fresh mass by 29-38% and root dry mass by 22-38% when compared to inoculated control plants that were not treated with *P. chlororaphis*.

3.4.4. Experiment 4: Effects of *Pseudomonas chlororaphis*, root-zone temperature, and root inoculation with *Pythium aphanidermatum* on leaf expansion

The area of leaf 12 was 47-49 cm² when *P. chlororaphis* was applied into the nutrient solution (Figure 3.4). Ten days later, when plants were inoculated with *P. aphanidermatum*, the area of leaf 12 in *P. chlororaphis*-treated and untreated plants did not differ significantly whether or not the roots were exposed to high pre-inoculation temperature. During the subsequent 12 days, leaf 12 progressively expanded in all treatments except in inoculated plants that were not treated *P. chlororaphis*. In respective plants of the high temperature predisposition treatment and in non-predisposed plants, leaf 12 ceased or almost
Figure 3.4. Area expansion of leaf 12 in pepper plants as a function of *Pseudomonas chlororaphis* (*Pc*) application in the nutrient solution, high temperature predisposition of the plants to Pythium root rot, and inoculation with *Pythium aphanidermatum* (*Pa*). *Pc* was applied 10 days before the roots were inoculated with *Pa*. The root zone was maintained at 23ºC for non-predisposed plants, but the temperature was increased to 33ºC for three days prior to inoculation with *Pa* for predisposition of plants to root rot. The experiment was repeated two times and results for one repetition are shown. Data are the means of 4 plants.
ceased to expand within 2 and 5 days after inoculation, which in each case coincided with initial root browning.

Treatment effects on the expansion of leaf 12 were based on values for area under the leaf expansion curve (AULEC) for the 12-day period immediately after plants were inoculated with *P. aphanidermatum*. Leaf areas on day 0 did not differ significantly among treatments and were used as base values for AULEC estimations. In plants not inoculated with the pathogen, the *P. chlororaphis* treatment significantly increased AULEC values from 332 to 485 units and from 319 to 381 units, respectively, in non-predisposed and predisposed plants. In inoculated plants that were not predisposed to root rot (constant 23°C), *P. chlororaphis* significantly increased the AULEC from 94 to 340 units. Respective values for plants that were predisposed at 33°C were 38 and 394 units. In general, expansion of leaf 12 was marginally lower following exposure of the roots to 33°C compared to a constant 23°C. Less than 40% of the roots were brown in *P. chlororaphis*-treated plants on day 12.

3.5. Discussion

The data provide a quantitative perspective of *P. chlororaphis* 63-28 in relation to progress of root rot and growth responses of peppers following high temperature predisposition compared to no pre-disposition of the roots to browning caused by *P. aphanidermatum*. *Pseudomonas chlororaphis* 63-28 applied in the plant nutrient solution at a final concentration of $10^7$ CFU mL$^{-1}$ was associated profusely and persistently with the pepper roots, delayed development of Pythium root rot, and markedly promoted growth of plants.
inoculated with *P. aphanidermatum* as well as of non-inoculated plants. In general, the agent performed as effectively in roots that were predisposed to precocious browning by the high temperature episode (described in Chapter two of this thesis) as in non-predisposed roots maintained at a constant 23°C.

The final density of *P. chlororaphis* applied in the nutrient solution 10 days before roots were inoculated with *P. aphanidermatum* was a critical factor affecting the ability of the agent to suppress root rot. A density of $10^7$ CFU mL$^{-1}$ solution delayed root browning more effectively than did the higher or lower densities when the root zone was kept at a constant 23°C, while densities of $10^6$, $10^7$ and $10^8$ CFU mL$^{-1}$ solution were equally superior in roots exposed to high temperature predisposition (Figure 3.1). Taken together, the data indicated that a final density of $10^7$ CFU mL$^{-1}$ solution was near optimal for root rot control.

Previous reports of inoculum density of *P. chlororaphis* in relation to Pythium root rot are lacking. However, Khan et al. (2003) and Chatterton et al. (2004) employed a final density of $10^7$ CFU *P. chlororaphis* TX-1 mL$^{-1}$ solution in pepper experiments, while Zheng et al. (2000) used $10^5$ CFU *P. chlororaphis* JZ-24 mL$^{-1}$ solution in cucumber studies. Densities near $10^6$ CFU cm$^{-3}$ rooting medium were evaluated in plants grown in rockwool (Rankin and Paulitz, 1994; McCullagh et al., 1996) and peat-based substrates (Gagné et al., 1993). My findings that the effectiveness of *P. chlororaphis* against root browning was much less when applied at $10^5$ or $10^4$ CFU mL$^{-1}$ compared to $10^6$ or $10^7$ CFU mL$^{-1}$ were consistent with the concept that a density threshold of a microbial agent is required for effective disease suppression (Raaijmakers et al., 1995). In this concept, small
changes in cell density above or below a critical threshold level markedly affect agent effectiveness, a phenomenon which may be related to quorum sensing in the microbial cell populations (von Bodman et al., 2003). Quorum sensing involves chemical communication among bacteria and allows bacterial populations to alter behavior in response to the number present in the community (Waters and Bassler, 2005). Effectiveness of *P. chlororaphis* may depend on maintaining adequate distribution of cells at densities above threshold levels in diverse niches on or within the roots. The finding that the effectiveness of *P. chlororaphis* was lower when applied at $10^8$ CFU mL$^{-1}$ compared to $10^7$ CFU mL$^{-1}$ in plants maintained at 23°C was possibly related to quorum quenching, and suggested that densities in excess of $10^7$ CFU mL$^{-1}$ solution may be counterproductive for root rot management.

The high density of *P. chlororaphis* 63-28 found in roots (approximately 1-6 x $10^6$ CFU g$^{-1}$ fresh roots) at 7 to 19 days after treatment in the two temperature regimes indicated that the final treatment density of $10^7$ CFU mL$^{-1}$ nutrient solution was sufficient for establishing a durable association of the strain with the growing root systems. The recovered densities were higher than reported for *P. chlororaphis* TX-1 by Khan et al. (2003) but similar to those found for strain TX-1 by Chatterton et al. (2004). The observation that a high density of *P. chlororaphis* was sustained for longer in roots inoculated with *P. aphanidermatum* than in non-inoculated roots agreed with findings of Chatterton et al. (2004) for *P. chlororaphis* TX-1, and supports the notion that diseased roots are more conducive to growth and survival of *P. chlororaphis* than are healthy
roots. The higher density of *P. chlororaphis* 63-28 in the diseased roots may be related to an increased partitioning of photosynthates to the roots and increased exudation of carbon compounds from the roots in response to root infection by *P. aphanidermatum* (Kamilova et al., 2006; Ortiz-Uribe, 2007). The moderate declines in density of *P. chlororaphis* observed at 19 days after treatment coincided with initial flowering of the peppers and so were possibly associated with flowering-related changes in host physiology. The estimates of *P. chlororaphis* CFUs should be interpreted with recognition that portions of the cell populations were possibly viable but not culturable (Kell et al., 1998).

The disease progress curves indicated that *P. chlororaphis* delayed root browning for 3 to 6 days following inoculation with *P. aphanidermatum*, but did not markedly affect the rate of increase in brown roots as indicated by the slopes of the browning curves. In inoculated plants that were not treated with *P. chlororaphis*, the pre-inoculation high temperature treatment advanced root browning by several days, but also did not generally affect the slopes and shapes of the browning curves. In effect, the *P. chlororaphis* treatment lengthened the biotrophic phase of Pythium root rot and delayed the necrotrophic phase, and the reverse was the case for pre-inoculation high temperature as reported in Chapter two of this thesis and Sutton et al. (2006). The clustering of the root browning curves of *P. chlororaphis*-treated plants at 9-12 days after inoculation (Figure 3.1) indicated that *P. chlororaphis* effectively re-mediated high temperature predisposition of the roots to precocious browning caused by *P. aphanidermatum*.
*Pseudomonas chlororaphis* 63-28 evoked major growth increases in the roots and shoots of pepper plants that were not inoculated with *P. aphanidermatum*. In summary, following an initial lapse of 7-10 to 10-13 days, the single application of *P. chlororaphis* progressively increased the growth parameters of the healthy vegetative peppers until the experiment was ended on day 22, despite the high nutrient availability in the root zone. Given the high density of *P. chlororaphis* in the roots at day 22, a potential existed for continued growth promotion without an additional treatment.

In plants inoculated with *P. aphanidermatum*, growth increases in response to *P. chlororaphis*, expressed on a percentage basis, were in several instances greater than in the non-inoculated plants. The *P. chlororaphis* treatment substantially mitigated the destructive effects of *P. aphanidermatum* on the growth components of the peppers, including the early cessation of leaf expansion (Figure 3.4). The major effects evoked by *P. chlororaphis* on root growth probably resulted in large part from the suppression of browning and growth reduction caused by the pathogen in the roots, but direct growth promotion may also have played a role. The markedly greater responses to *P. chlororaphis* observed for leaf area than for shoot fresh mass and dry mass in the inoculated plants suggest that the plants partitioned proportionately more carbon resources to the leaves than the stems (Figure 3.4 and Table 3.2).

Growth of the young leaf (leaf 12) was a sensitive marker of root infection by *P. aphanidermatum* and of plant growth promotion by *P. chlororaphis*, but not of stress associated with the high temperature episode in the root zone (Figure
3.4). In the absence of *P. chlororaphis*, the cessation or near cessation in growth of leaf 12 in less than two days after inoculation in plants of the high temperature regime and within five days for plants at a constant 23°C, contrasted with the continued rapid growth of the equivalent leaf in the non-inoculated control plants (Figure 3.4). The findings for leaf 12 were consistent with observations in hydroponically-grown snapdragons inoculated with the same strain of *P. aphanidermatum* (Sutton et al., 2006; Ortiz-Uribe, 2007). Growth measurements of leaf 12 also provide a relatively non-intrusive means for quantitative tracking of the effects of *P. chlororaphis* on canopy development in healthy and inoculated plants. The measurements did not, however, detect changes induced in the roots by the high temperature treatment which resulted in predisposition of inoculated roots to browning that was reported in Chapter two of this thesis. While the temperatures, pH, dissolved oxygen levels, and composition of the nutrient solution in the single-plant hydroponic units were representative of those in pepper crops in Ontario, several other conditions were different, and may warrant consideration should *P. chlororaphis* 63-28 be developed for commercial use. The high turbulence of the nutrient solution arising from aeration bubblers in the hydroponic units probably contrasts with turbulence of solutions of commercial crops that percolate through rooting matrices such as rockwool and coconut fiber (Sutton et al., 2006). Zoospores of *P. aphanidermatum* readily lose flagella and thus motility under turbulent conditions (Chatterton et al., 2004; Sutton et al., 2006), but possible effects of turbulence on *P. chlororaphis*, such as its ability to associate with roots, have not been reported. Susceptibility to Pythium root rot is
generally high when plants are young and vegetative, as in the present studies, but generally decreases with age, notably at the flowering and fruiting stages, although young roots are highly susceptible at all stages (Kamoun et al., 1999; Martin and Loper, 1999; Sutton et al., 2006; Ortiz-Uribe, 2007). The intensity of PAR, a key factor influencing productivity and levels of carbon compounds in the root zone and top growth (Ortiz-Uribe, 2007), was much lower in the growth room compared to daytime levels in commercial greenhouses. The abrupt exposure of roots to a high zoospore density of *P. aphanidermatum* during inoculations probably differed substantially from zoospore density dynamics in hydroponic crops. Implications of the foregoing and other factors in the epidemics and control of Pythium root rot in commercial crops were considered earlier (Sutton et al., 2006).

The persistent association of *P. chlororaphis* 63-28 with the roots, the suppression of root browning, and the plant growth promotion by the agent in the pepper plants suggest that the strain has substantial potential for enhancing the health and productivity of hydroponic pepper crops. The strong growth promotion in the absence of *P. aphanidermatum* may justify its use as a root-zone inoculant in healthy crops, while the strong suppression of root rot underscored its value as a biological control agent. Tests are needed under representative crop conditions to determine the performance of strain 63-28 in relation to pepper productivity, such as earliness of fruiting and marketable fruit yield, and as functions of treatment protocols such as timing and mode of application. The growth effects of strain 63-28 may require that adjustments be made in levels of nitrogen or other
elements in the plant nutrient solution. Results of the present study and beneficial effects reported previously (Gagné et al., 1993; McCullagh et al., 1996; Liu et al., 2003; Liu et al., 2007; Corrêa et al., 2010) suggest that strain 63-28 has substantial value as a versatile inoculant and control agent against Pythium root rot in a range of hydroponic crops.
Chapter 4. General discussion

For decades, outbreaks of PRR and several other diseases in greenhouse crops have been attributed to enhanced pathogen activity when crops are stressed by environmental factors (Jarvis, 1992; Sutton et al., 2006). The purported role of stress factors is supported in part by anecdotal reports from commercial greenhouses and in some instances to results of scientific investigations of environmental factors in relation to disease increase. The present report is the first in which quantitative relationships of a stress factor, (i.e. high temperature), and disease progress were determined as functions of time in a hydroponic greenhouse crop.

High temperature is well established as a stress factor that favors increase in severity of PRR and certain other diseases in sweet peppers and other crops. Previous studies generally focused on effects of constant temperature levels on PRR in plants already inoculated with *P. aphanidermatum* so that it was not possible to discriminate between temperature effects on the host, the pathogen, or the host-pathogen interaction. In most previous studies temperature effects also were confounded by other variables such as DO levels in the nutrient solution and the type of rooting medium used. In commercial greenhouses periods of high temperature are episodic. For example, episodes of daily temperatures that are sufficiently high to induce stress responses in the crop on intermittent days or on one or more sequences of days. Patterns of high temperature within episodes generally differ so that different intensities of stress responses may be expected. Questions arise such as “are stress responses to a
series of high temperature episodes in some way cumulative?” and “how persistent is the stress response when periods with no stress level temperatures resume?” These relationships are fundamental to understanding how high temperature stress affects progress of PRR and for refining a predictive model that can be used as a basis for timing management measures against PRR. The present investigation is the first to provide a quantitative measure of episodes of high root-zone temperature in relation to the susceptibility of a crop to PRR. It also unequivocally demonstrates for the first time that episodes of high temperature can predispose a crop to PRR, and to provide an understanding of the quantitative relationships between the duration of such episodes and subsequent progress and severity of PRR.

A further principal contribution of the present work is the demonstration that *P. chlororaphis* 63-28 substantially re-mediated predisposition to root rot when the pepper host is grown with a root zone temperature that does not predispose roots to PRR (i.e. 23°C) and also under particular conditions of high temperature stress (i.e. 72 h at 33°C). While root rot (browning) ultimately progressed in plants inoculated with *P. aphanidermatum*, whether or not they were treated with *P. chlororaphis*, it is important to consider that the roots were abruptly exposed to a very high density of zoospores when inoculated and that this density was far greater than would normally be expected in a hydroponic crop. Biocontrol effectiveness of *P. chlororaphis* can be expected to last much longer when build-up of zoospore populations is more gradual as normally occurs in root zones of commercial hydroponic crops.
Data in this thesis confirmed the ability of strain *P. chlororaphis* 63-28 to promote foliage and root growth in healthy as well as diseased peppers. The study also reports for the first time that leaf expansion is a good nonintrusive indicator of root stress that has the potential to allow remote monitoring of PGPR effectiveness in promoting growth in the presence and absence of the pathogen, under moderate or high temperature conditions.

The observations in chapters 2 and 3, taken together, contribute to a rationale for employing *P. chlororaphis* 63-28 against PRR in hydroponic pepper crops. A major advantage of strain 63-28 is that it is as effective in roots with increased susceptibility to high temperature stress as in non-stressed roots. This finding might not hold for other biological control agents or for chemical control measures. The importance of treating roots with strain 63-28 prior to high temperature episodes is underscored by the risk of explosive development of PRR should one or more high temperature episodes occur. Prediction of hot weather is thus important for managing PRR and timing treatments with beneficial microbes. The data of chapter 2 provide a guide to the temperature levels and durations that might function as thresholds for remedial actions such as introducing cooler nutrient solution into the root zone. An ability to predict such temperature conditions is needed for timing applications of 63-28 or other beneficial microbes into the nutrient solution. Observations of the present and previous studies (Khan et al., 2003: Chatterton et al., 2004) underscore the need for treating roots with 63-28 and other strains of *P. chlororaphis* at least 3 to 7 days before roots are exposed to *P. aphanidermatum*. The concept of using the
rate of leaf expansion as a sensitive marker of PRR and of remediation by beneficial agents warrants further consideration.

*Pseudomonas chlororaphis* 63-28 has the advantage that it is ecologically well adapted to peppers and able to persist in the roots in sufficiently high densities to markedly suppress PRR. For example, high populations of the bacterium were recovered from the roots of vegetative stage peppers until at least 19 days after it was applied into the root zone at a final density of $10^7$ CFU mL$^{-1}$ nutrient solution. Thus, the findings imply that the application of $10^7$ CFU mL$^{-1}$ nutrient solution achieves a critical threshold level for quorum sensing in the bacterial populations and for effective disease suppression and plant growth promotion (Raaijmakers et al., 1995; Teplitski et al., 2000; von Bodman et al., 2003). The results also suggest that treatments at about 3-week intervals might be sufficient for continued suppression of PRR throughout the pepper crop cycle. In-crop testing is needed however to confirm the adequacy of such a treatment program and perhaps to refine the application intervals for optimal results.

The data obtained in this thesis have the potential to be integrated with other relevant information to develop protocols for applying *P. chlororaphis* and other measures in pepper crops for season-long protection against PRR. Given that *P. aphanidermatum* and other *Pythium* spp. are often continuously present in hydroponic systems, it is almost certainly important to initiate management measures against PRR at an early stage of crop growth, such as at the time of transplanting into the production greenhouse. Early treatment is especially
important for delaying PRR development and the transition from biotrophy to necrotrophy in fast-growing vegetative peppers. Protocols for using *P. chlororaphis* and other measures against PRR are best founded on an understanding of relevant epidemiological factors that affect PRR i.e. scientific knowledge of the host, the pathogen, and the crop environment including microclimatic variables, physical parameters of the hydroponic system and the indigenous microflora of the root zone. For commercial acceptance the protocols have to be practical and cost-effective. All measures have to prove profitable or the growers will quickly cease to use them.

The data reported in this thesis are fundamental, having been obtained in replicated experiments conducted under rigorously controlled conditions, so have applications for PRR control in many kinds of hydroponic systems employed around the world. The quantitative information thus is relevant for use in hydroponic crops in other cool temperate climates such as in parts of Europe and Asia. As in Southwestern Ontario many systems used in these areas employ enclosed structures with sophisticated computerized environmental control and can be operated year-round. The data are expected to also have applications in hydroponic systems used in warm temperate and tropical climates. For example, in the humid tropics of the Brazilian state of Pará, the main hydroponic crops (lettuce, various herbs and ornamentals) are grown in “casas de vegetação” which are generally open-sided wood-framed structures with roof coverings of plastic sheeting and horticultural mesh to provide protection against harsh weather conditions such as tropical rains and intense heat and sunlight (Sutton et
Root-zone temperatures are often high and frequently favor severe PRR.

The continued and increasing public demand for pesticide-free foods, and increased restrictions on pesticide use by regulatory agencies, have increased the need and urgency for alternative means to manage PRR in hydroponic crops. The findings in this thesis justify further investigations of *P. chlororaphis* 63-28 as a key component of integrated pest management programs in commercial greenhouses. For pursuing this goal, information is needed on factors such as the compatibility of *P. chlororaphis* 63-28 with other nutrient solution treatments such as ozonation, ultra-filtration, fungicides, oxygenation, cool water fluxes, and other microbial agents. Methods for introducing the bacteria into different kinds of hydroponic systems (e.g. re-circulating systems with rockwool or coir; floating systems) also require attention so as to assure adequate treatment throughout the root zone of the crop. The overall goal is normally to minimize densities of the pathogen in the root zone and to slow down the progress of PRR sufficiently to avoid yield losses. The present data suggest that, should PRR remain mild or absent, treatments with *P. chlororaphis* 63-28 may be expected to enhance crop growth and productivity, as was found in other crops (Liu et al., 2007). As with other pseudomonads, an urgent research task for commercialization of *P. chlororaphis* 63-28 is to develop formulations of the bacteria that allow the cells to effectively survive and remain active during product distribution and storage (Weller, 2007).
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