Quantitative relationships of *Pseudomonas chlororaphis* 63-28 to Pythium root rot and growth in hydroponic peppers

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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 23ºC except when roots were exposed to 33ºC for three days before inoculation to induce predisposition to root rot. At constant 23ºC (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. 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 seven 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*. We conclude that *P. chlororaphis* 63-28 has substantial potential for managing the disease regardless of predisposition.

Key words: *Capsicum annuum*, *Pythium aphanidermatum*, disease predisposition, biological control, leaf expansion.

RESUMO

Relações quantitativas de *Pseudomonas chlororaphis* 63-28 com a podridão radicular causada por *Pythium* e ao crescimento de pimentão hidropônico

A supressão da podridão de raiz (*Pythium aphanidermatum*) e a promoção de crescimento de pimentão hidropônico por *Pseudomonas chlororaphis* 63-28 foram avaliadas em plantas predispostas ou não ao patógeno. O bioagente foi introduzido na solução nutritiva 10 dias antes das raízes serem inoculadas com o patógeno. A zona radicular foi mantida a 23ºC, exceto nos três dias antes da inoculação quando as plantas foram expostas a 33ºC. Na temperatura de 23ºC (sem predisposição), *P. chlororaphis* na concentração de 10^7 UFC mL^{-1}, aplicada na solução nutritiva, atrasou o aparecimento dos sintomas da doença. As concentrações de 10^6, 10^7 e 10^8 UFC mL^{-1} do bioagente foram mais eficientes em controlar a doença nas plantas inoculadas com o patógeno. A densidade do bioagente nas raízes, nos dois regimes de temperatura, variou de 5,88 a 6,45 log da UFC g^{-1} de raiz fresca, do sétimo ao 19º dia após a aplicação de 10^7 UFC mL^{-1}. Nas plantas inoculadas ou não, o bioagente atrasou o aparecimento do escurecimento radicular, remediou a predisposição a doença e incrementou o desenvolvimento das plantas. A expansão foliar foi um indicador adequado para a podridão de raiz e a remediação com *P. chlororaphis*. Conclui-se que *P. chlororaphis* 63-28 tem potencial para o manejo da podridão da raiz em sistemas hidropônicos, independentemente da predisposição.

Palavras-chave: *Capsicum annuum*, *Pythium aphanidermatum*, controle biológico, expansão foliar, predisposição a doença.

INTRODUCTION

Sweet peppers (*Capsicum annuum* L.) are produced all year round in Ontario greenhouses by means of sophisticated hydroponic systems. The pepper plants are normally grown in slabs of rock wool or coconut fiber, 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). Sopher & Sutton (2009) confirmed that high root-zone temperature favors rapid development of root rot and found that roots of healthy peppers were 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 to 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. Taken together, 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 & Bettiol, 2009). 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 & Bettiol, 2009; Corrêa et al., 2010). Those with superior effectiveness included strains of *Bacillus subtilis*, *Bacillus cereus*, *Streptomyces griseoviridis*, *Pseudomonas fluorescens*, *Pseudomonas corrugata*, *Pseudomonas chlororaphis*, *Comomonas acidovorans*, *Clonostachys rosea*, and *Trichoderma harzianum* (Rankin & Paulitz, 1994; McCullagh et al., 1996; Paulitz & Bélanger, 2001; Zheng et al., 2000; Liu et al., 2003; Liu et al., 2007). Strains of *Pseudomonas chlororaphis* (= *Pseudomonas aureofaciens*) (Johnson & 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 to 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 that high root zone temperature predisposes pepper plants to Pythium root rot (Sopher & Sutton, 2009) 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: i. 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*; ii. to determine the optimum density of *P. chlororaphis* 63-28 inoculum for root rot control; iii. 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*; iv. to examine quantitative effects and interactions of *P. chlororaphis* 63-28, root-zone temperature regimes, and *P. aphanidermatum* on growth parameters of vegetative peppers; v. to assess leaf expansion as a minimally intrusive means to estimate effectiveness of *P. chlororaphis* 63-28 against root rot.

MATERIAL AND METHODS

Hydroponic pepper plants

Plants of sweet pepper cv. Cubico (De Ruiter Seeds Inc., Columbus, OH, USA) were grown on a bench in a growth room as described by Sopher & Sutton (2009). In brief, 5-week old seedlings in rock wool 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 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 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 rock wool 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).

Control of high root zone temperature

The hydroponic units were placed in temperature-controlled water baths to control root zone temperature as described by Sopher & Sutton (2009). The baths were positioned on the bench in the growth room.

Inoculum production and application of *Pseudomonas chlororaphis*

Pseudomonas chlororaphis strain 63-28 was used in all 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 $MgSO_4$. 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 10^9 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 10^7 CFU mL^{-1} unless otherwise indicated.

Pythium inoculum and inoculations

The protocols used for maintenance and growth of *P. aphanidermatum* and for inoculating pepper roots were described by Sopher & Sutton (2009). 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 x 10^3 zoospores mL^{-1} for 30 min, and returned to the unit.

Density of *Pseudomonas chlororaphis* in roots

Numbers of *P. chlororaphis* cells associated with pepper 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 roots of all orders and depths in the hydroponic units. Suspensions of the ground tissues were serially diluted, spread onto TSA medium in Petri dishes, and incubated at 25˚C for 48 - 72 h. Colony counts were used to estimate CFU g^{-1} fresh roots.

Root colonization by *Pythium* 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 six-cm diameter 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%). Discoloration was usually gray-brown or reddish-brown. Mid-point values of scale increments were used for data analysis.

Plant growth analysis

In destructive harvests, the height of each plant was measured from the rock wool 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 an area meter (model LI-3000, LI-COR, Lincoln, NE, USA) was used to measure total leaf area per plant. All roots exterior to the rock wool 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.

Experiment 1: Progress of Pythium root rot in relation to density of *P. 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 (Sopher & Sutton, 2009) 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.

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*

The nutrient solution of hydroponic pepper plants was treated with *P. chlororaphis* at a final density of 1 X 10^7 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 three, six and nine 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).

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 three, six, nine and 12 days after inoculation.

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 for experiment 2. 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.

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. There were four replicate plants per treatment in experiments 1, 3 and 4 and three in experiment 2. Data were analyzed 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.

RESULTS

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 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 two 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. Linear equations for the slopes ranged from Y=16.8x-57.6 to Y=18x-112.8.

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 zero or three h after inoculation, respectively 12%, 46% and 70% for samples taken at six, nine and twelve 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.

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 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}
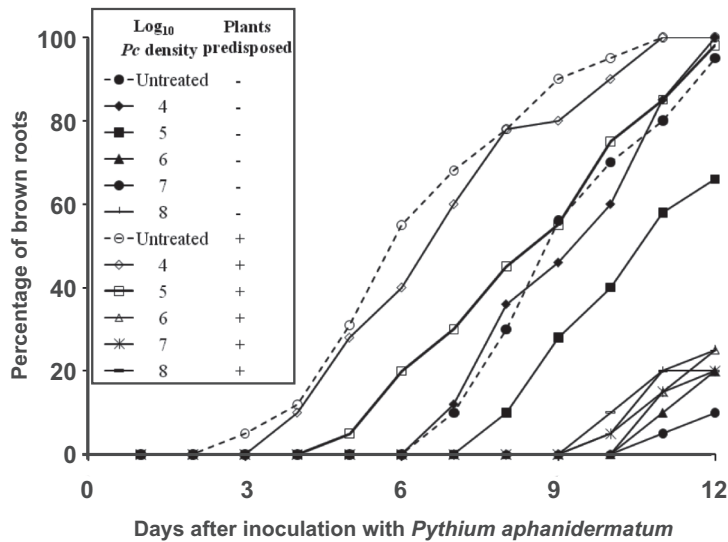
FIGURE 1 - Root browning progress curves in hydroponic pepper plants inoculated with *Pythium aphanidermatum* (*Pa*) as a function of density of *Pseudomonas chlororaphis* 63-28 (*Pc*) applied in the nutrient solution and high temperature predisposition of the plants to Pythium root rot. *Pc* was applied 10 days before the roots were inoculated with *Pa*. The root zone was maintained at 23ºC for non-predisposed plants but the temperature was increased to 33˚C for three days prior to inoculation to predispose plants to root rot.
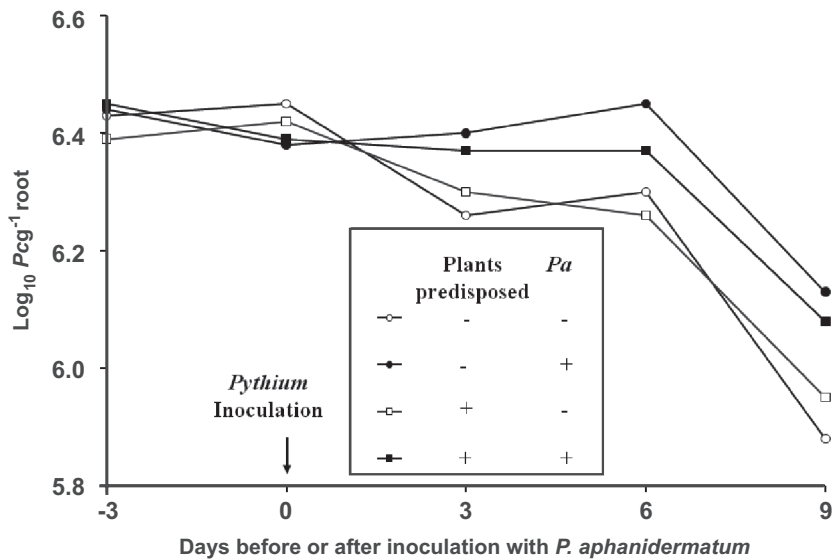

FIGURE 2 - Estimated density of *Pseudomonas chlororaphis* 63-28 (*Pc*) in hydroponic pepper roots as a function of time after application, high temperature predisposition of the plants to root rot, and root inoculation with *Pythium aphanidermatum* (*Pa*). *Pc* was applied in the nutrient solution at 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 33˚C for three days prior to inoculation to predispose plants to root rot.

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 three, six and nine 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 of experiment 2 did not differ significantly from those of the same treatments and conditions in experiment 1. *Pseudomonas chlororaphis* delayed initial browning of inoculated roots by seven days for plants that were exposed to 33˚C compared to five days for plants at a constant 23˚C. In each instance, browning did not exceed 30% when the experiment ended at 12 days after inoculation (22 days after *P. chlororaphis* was applied).

Experiment 3. Effects of *Pseudomonas chlororaphis*, root zone temperature, and root inoculation with *Pythium aphanidermatum* on plant growth parameters

    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 *P. aphanidermatum*, or at 13 days (three days after inoculation), but significant effects were found at 16, 19, and 22 days (six, nine and 12 days after inoculation). Significant increases in total plant leaf area were first observed at 10 days after the *P. chlororaphis* treatment (the day of inoculation). At 19 days (nine days after inoculation), the shoots and root systems of plants treated with *P. chlororaphis* generally appeared larger than those of untreated plants, regardless of the root zone temperature regime or whether the roots were inoculated with *P. aphanidermatum* (Figure 3). Root rot was markedly less severe in *P. chlororaphis*-treated plants than in untreated plants in each temperature regime.

Plant height

    Plants that were not treated with *P. chlororaphis* but inoculated with *P. aphanidermatum* were significantly shorter than plants of all other treatments at six and nine days after inoculation (16 and 19 days after *P. chlororaphis*

treatment) but not at earlier sampling times. At nine days after inoculation, plant height in given treatments of the two temperature regimes did not differ significantly (Table 1). Plants that were untreated and not inoculated with *P. aphanidermatum* averaged 26 cm in height. The pathogen suppressed the height of untreated plants by 8-10%. The *P. chlororaphis* treatment increased plant height by 18-19% in plants not inoculated with the pathogen and by 10-11% in the inoculated plants.

Total plant leaf area

    The mean total leaf area per plant was 270 cm^2 when *P. chlororaphis* was applied in the nutrient solution. After seven days, when the high temperature treatment was initiated, the respective mean leaf areas of *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 *P. aphanidermatum*, leaf area was significantly greater in *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 (nine days after inoculation) in plants not treated with *P. chlororaphis*, leaf area was 21-25% lower in inoculated compared to non-inoculated plants (Table 1). By 19 days following application, *P. chlororaphis* increased leaf
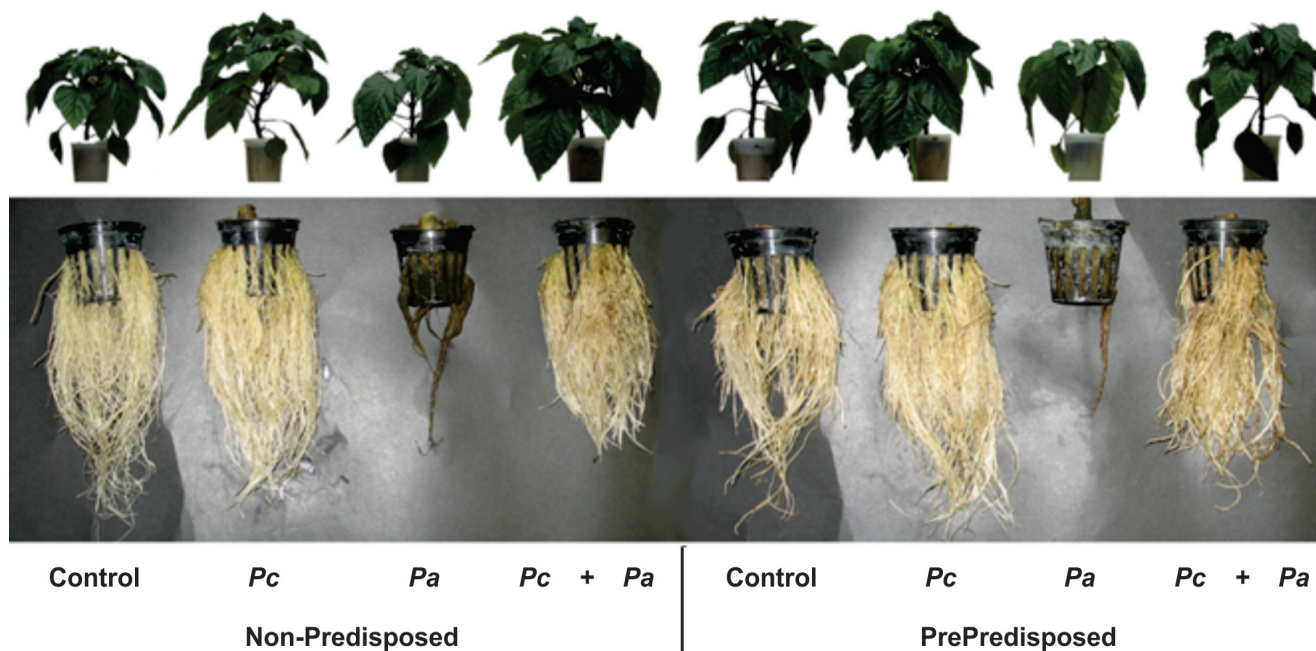

FIGURE 3 - Pictorial comparison of the effects of *Pseudomonas chlororaphis* 63-28 (*Pc*) and inoculation of the roots with *Pythium aphanidermatum* (*Pa*), separately and in combination, in pepper plants that were not predisposed or predisposed to Pythium root rot. Plants were photographed at nine days 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 23ºC and the temperature was increased to 33˚C for three days prior to inoculation with *Pa* for predisposing the plants.

TABLE 1 - Growth components of hydroponic peppers that were treated or not treated with *Pseudomonas chlororaphis* (*Pc*), predisposed or not predisposed to root rot by 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*)

Pc	Plants predisposed	*Pa*	Plant height (cm)	Total leaf area (cm^2)	Shoot fresh mass (g)	Shoot dry mass (g)	Root fresh mass (g)	Root dry mass (g)
-	-	-	26.0c	1404c	62.9c	5.4c	11.8b	1.0 b
+	-	-	31.0a	1727a	70.6a	6.3a	13.8a	1.2a
-	-	+	23.3d	1114d	54.1d	4.8d	10.1c	0.9c
+	-	+	28.3b	1621ab	61.6c	5.4c	13.0ab	1.1ab
-	+	-	25.7c	1439c	60.4c	5.3c	11.7b	1.0b
+	+	-	30.7a	1695a	72.6a	6.4a	13.6a	1.2a
-	+	+	23.7d	1075d	51.3d	4.8d	9.1c	0.8c
+	+	+	28.7b	1513bc	62.2bc	5.6bc	12.6b	1.1b

Treatment means in a column followed by the same letter do not differ significantly by ANOVA (P=0.05; LSD).

area by 18-23% in plants not inoculated with the pathogen and by 45-58% in the inoculated plants.

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 among treatments. Values of the respective variables also were not significantly different on day 13 (three days after inoculation). At day 16 (six 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 respectively increased root fresh mass and dry mass by 14% and 11% 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 23ºC.

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 1).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%. 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*.

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 cm^2 when *P. chlororaphis* was applied into the nutrient solution (Figure 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 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 23ºC), *P. chlororaphis* significantly increased the AULEC from 94 to 430 units. Respective values for plants that were predisposed at 33ºC were 38 and 394 units. In general, expansion of leaf 12 was
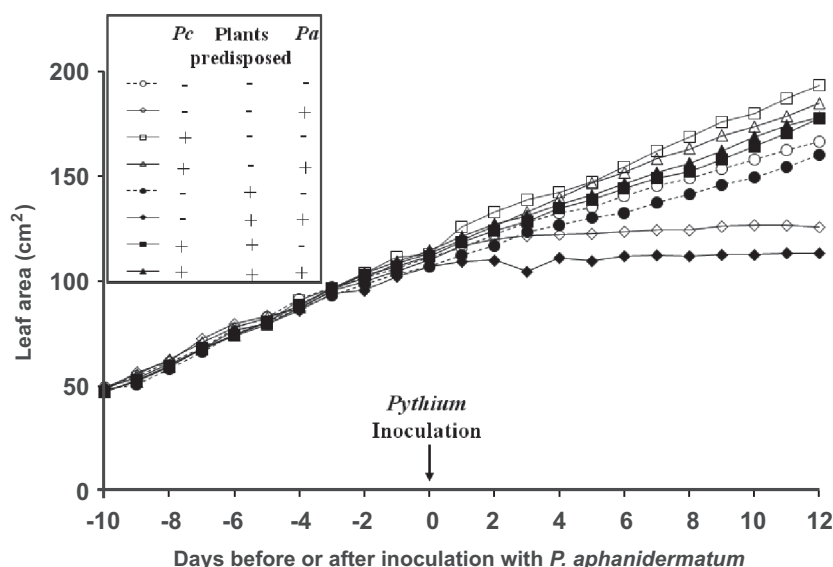
FIGURE 4 - Area expansion of leaf 12 in pepper plants as a function of *Pseudomonas chlororaphis* (*Pc*) application in the nutrient solution, high temperature predisposition of the plants to Pythium root rot, and inoculation with *Pythium aphanidermatum* (*Pa*). *Pc* was applied 10 days before the roots were inoculated with *Pa*. The root zone was maintained at 23 ºC for non-predisposed plants but the temperature was increased to 33˚C for three days prior to inoculation with *Pa* for predisposition of plants to root rot.

marginally lower following exposure of the roots to 33ºC compared to a constant 23ºC. Less than 40% of the roots were brown in *P. chlororaphis*-treated plants on day 12.

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* and of non-inoculated plants. In general, the agent performed as effectively in roots that were predisposed to precocious browning by the high temperature episode (Sopher & Sutton, 2009) 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 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 rock wool (Rankin & Paulitz, 1994; McCullagh et al., 1996) and peat-based substrates (Gagné et al., 1993). Our 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 & 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 sensing, 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 seven 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 three to six 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 (Sutton et al., 2006; Sopher & Sutton, 2009). The clustering of the root browning curves of *P. chlororaphis*-treated plants at 9–12 days after inoculation (Fig. 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 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 4 and Table 1).

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 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 23ºC, contrasted with the continued rapid growth of the equivalent leaf in the non-inoculated control plants (Figure 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 (Sopher & Sutton, 2009).

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 rock wool 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 & 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 (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.

ACKNOWLEDGEMENTS

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