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Selective enhancement of indigenous fluorescent pseudomonads via chemical amendments to recycled irrigation water: An integrated approach to managing diseases and promoting plant growth in soilless agriculture

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"The most beautiful experience we can have is the mysterious. It is the fundamental emotion that stands at the cradle of true art and true science.
Whoever does not know it and can no longer wonder, no longer marvel, is as good as dead, and his eyes are dimmed".

--Albert Einstein

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ABSTRACT OF THE DISSERTATION

Selective enhancement of indigenous fluorescent pseudomonads via chemical amendments to recycled irrigation water: an integrated approach to managing diseases and promoting plant growth in soilless agriculture

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Zoosporic root-infecting pathogens are the most destructive organisms in recirculating hydroponic systems. Strategies proposed for eliminating these pathogens from recycled nutrient solution include: filtration, sedimentation, chlorination, ozonation, heat, UV light, and the application of antimicrobial chemicals or biocontrol agents. The latter strategy has been investigated extensively but inconsistent performance in disease abatement following their employment has been a problem due to the inability of the biocontrol agent to maintain a critical threshold population necessary for sustained activity.

Amendment of the recycled nutrient solution with specific carbon substrates (i.e., synthetic and natural nitrification inhibitors) resulted in the selective enhancement of indigenous pseudomonad populations to the presumed threshold levels. We verified the ubiquity of that response in several hydroponic systems with different host plants and, most significantly, we document, for the first time, significant and sustained disease abatement. A combination of traditional (plate counting) and culture-independent methods (T-RFLP and Biolog™ assay) suggested that nitrification inhibitors selectively increased *P. putida* and reduced bacterial diversity in the nutrient solution. The enhanced indigenous pseudomonad also colonized roots. An introduced biocontrol agent, *P. fluorescens* Pf5, showed no competitive advantage over the native pseudomonad. Additionally, we demonstrated that the active ingredient in the nitrification inhibitors exerted direct antifungal activity whereas inert ingredients had indirect role in disease suppression via the enhanced pseudomonad population. In general, amendment of the recycled nutrient solution with nitrification inhibitors had a stabilizing effect on pH and electrical conductivity and promising positive effects on plant growth and yield.

Cumulatively, our results support the hypothesis that it is possible to modify the environment to make it more conducive to the survival of indigenous bacteria. Many, if not most, habitats already contain prospective members who, if enhanced, could function as biocontrol agents.

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GENERAL INTRODUCTION

The production of vegetable and ornamental crops in hydroponic systems is of interest for several reasons. In combination with greenhouses, it is highly productive, conservative of water and land, and protective of the environment. It also meets the growing demand by consumers for sound, biologically-based crop management practices. Hydroponics provides these new requirements. The switching over from the soil to hydroponics results in decreased application of pesticides and other toxic agrochemicals used in conventional culture in soil to disinfest and to control soilborne plant pathogens [27]. Greenhouse growers, using open hydroponic systems, also are facing strict governmental regulations concerning the discharge of spent nutrient solution in order to abate ground water pollution. At the same time the use of recaptured irrigation water by the agricultural/horticultural industry is increasing due to pressure by water purveyors and governmental agencies to reduce water usage [5]. However, completely closed recycled systems in which the nutrient solution is recycled may be a solution to the problem of pollution and water conservation.

Although the use of recycled irrigation water will reduce water usage and mitigate nutrient runoff from nursery production sites, serious grower concerns exist regarding the spread of phytopathogenic and/or deleterious microorganisms in the recycled water. Inhibition of deleterious microorganisms in recycled water has been attempted using various methods including, heat treatment, ozone, UV radiation, slow sand filtration and chemicals such chlorine [1, 5, 25, 26, 41, 42]. These methods are known to reduce the pathogenic microflora but may also have adverse effects due to the suppression of some other beneficial resident microflora [5] and the creation of a biological vacuum that can lead to a more rapid and increased spread of root-infecting pathogens if the latter are introduced after disinfestations. The primary root infecting pathogens are zoosporic, many of which belong to the genera *Pythium*, *Phytophthora*, *Plasmopara* and *Olpidium*. These microorganisms produce a motile stage knows as zoospores which are favored by an aquatic environment [31].

Nowadays a change in the way of thinking about disinfection and disinfestations has emerged. Methods based on eliminating all pathogens and sterilizing the nutrient solution has been questioned. It is perhaps not advantageous to kill all life in solution; there is a certain microflora present which may plays a role in suppressing disease [17, 22, 23,

24]. Therefore, certain micro-organisms are able to suppress diseases. If they are eliminated, the nutrient solution loses it suppressiveness and a rapid outbreak of a disease can occur if a pathogen is accidentally introduced into the biological vacuum.

Some of the resident bacteria are referred to as plant growth promoting rhizobacteria (PGPR). These rhizosphere bacteria have been demonstrated to stimulate plant growth and improve crop productivity [10, 11, 16]. Known mechanisms for the latter include the production of hormones (such as indol acetic acid, IAA) [15], solubilization of phosphate, or, more generally, increasing mineralization [13]. Additionally, increased plant productivity has also been attributed to the suppression of deleterious and soilborne pathogens by these resident rhizosphere bacteria [28]. These latter bacteria, in particular the fluorescent *Pseudomonas* species, act by producing antibiotics, siderophores, biosurfactants, and/or nutrient competition [19]. In the 1990's, it was also demonstrated that these bacteria, employed as biocontrol agents or PGPR, could induce systemic resistance (ISR) in plants against some pathogens [14, 18, 20, 35, 36, 37, 38].

In the conventional integrated disease management (IDM) studies conducted to date, all strategies combine biological and chemical control in a separate manner. Recently, there has been a growing interest in combining microbial agents with other chemical components to enhance their activity against certain soilborne plant pathogens [30]. Microbial inoculants (especially *Pseudomonas s*pp.) can play an important role in IDM systems. Unfortunately, despite the attractiveness and potential for success of this strategy, biological control of root diseases in hydroponic systems has not been realized. Efficacy of the bioinoculants in disease abatement has been inconsistent, partially due to the variable *in situ* production or inactivation of bacterial metabolites supposedly responsible for disease control and/or the inability of the biocontrol bacterium to maintain a critical threshold population necessary for sustained biocontrol activity [6, 7, 33]. The activities and population densities of introduced PGPR bacteria generally decline with time after their application, thus making the beneficial response of short duration. To enhance and extend the desired responses, the environment needs to be altered to selectively favor the activities of the introduced biocontrol agent [2, 3, 4]. Lack of specific nutrients has been identified as a limiting factor to the growth of the specific microbial populations in various plant habitats [9] but this can be overcome by the addition of specific substrates to the environment which are utilized selectively by the introduced microbe employed as a

biocontrol agent. For example, specific nutritional amendments such as chitin applied as an amendment along with chitinolytic *Bacillus cereus* strain 304, increased the population size of this bacterium on peanut leaves, resulting in significant disease control [12]. Amino acids were used as an amendment to enhance the population size of an antagonistic nonpathogenic bacterial strain on apple fruits, resulting in improved biological control of blue mold on ripe apples [8]. Methionine increased the population size of the biological control agent *Pseudomonas putida* AP-1 in soil, and the suppression of *Fusarium* wilt on tomato [43]. Under glasshouse conditions soil amendment with zinc and/or glucose in combination with *Pseudomonas aeruginosa* strain IE-6S^{+} or *P. fluorescens* strain CHAO greatly reduced *Macrophomina phaseolina* populations in the rhizosphere of tomato plants [29]. Exogenous application of, a salicycate carbon source, increased the population size of a salicylate-catabolizing *P. putida* strain in the phyllosphere of beans [39]. Endogenous provision of mannityl opines by mannityl opine-producing transgenic tobacco also increased the population size of *Pseudomonas syringae* Cit7(pYDH208) in the phyllosphere [40]. Various exotic carbon sources were tested by Paulitz [21] under greenhouses conditions to enhance strain *Pseudomonas fluorescens* strain 63-28, but the bacteria could not utilize them. Strain 63-28 could utilize mannitol, sorbitol and trehalose but no biocontrol activity was increased. Stanghellini and Miller, [32] showed that the amendment of olive oil to the recirculating nutrient solution of hydroponically grown peppers, stimulated the metabolic activity (i.e., rhamnolipid production) and population density of an introduced strain (R4) of *P. aeruginosa* which resulted in the control of *Phytophthora capsici*.

Among all the strategies, described in literature, used to enhance the survival of bacteria, few reported studies on hydroponic systems and more importantly rarely described new methods to enhance native biocontrol bacteria. Hydroponic studies on the possibility to use a single commercial product that exhibit biological and chemical properties (bi-functional) and thus combine different control strategies has never been investigated. This new approach could help to discourage the development of disease, to maintain pesticides and other chemicals at reasonable levels, to reduce the risks to human health and to the environment, and most importantly could encourage natural disease control mechanisms.

The overall objective of this thesis was to evaluate alternatives integrated disease management (IDM) strategies for zoosporic pathogens in recirculating systems. Specifically, this thesis presents data regarding (i) the qualitative and quantitative assessment of changes in the resident, as well as introduced, bacterial populations in recycled nutrient solutions of hydroponically grown plants amended with specific chemicals, (ii) the efficacy of these amendments for management of root disease of pepper and cucumber caused by *Phytophthora capsici* and *Pythium aphanidermatum,* respectively, and (iii) the mechanism(s) associated with selective enhancement of specific bacterial populations and disease suppression following chemical amendment of the recycled nutrient solution.

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CHAPTER 1

SELECTIVE ENHANCEMENT OF THE FLUORESCENT PSEUDOMONAD POPULATION AFTER AMENDING THE RECIRCULATING NUTRIENT SOLUTION OF HYDROPONICALLY-GROWN PLANTS WITH A NITROGEN STABILIZER

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(Submitted to Microbial Ecology)

ABSTRACT

Fluorescent pseudomonads have been associated, via diverse mechanisms, with suppression of root disease caused by numerous fungal and fungal-like pathogens. However, inconsistent performance in disease abatement, following their employment, has been a problem. This has been attributed, in part, to the inability of the biocontrol bacterium to maintain a critical threshold population necessary for sustained biocontrol activity. Our results indicate that a nitrogen stabilizer (N-Serve®, Dow Agrosciences) selectively and significantly enhanced, by 2-3 order of magnitude, the resident population of fluorescent pseudomonads in the amended (i.e., 25 μg ml^{-1} nitrapyrin, the active ingredient) and recycled nutrient solution used in the cultivation of hydroponically-grown gerbera and pepper plants. *Pseudomonas putida* was confirmed as the predominant bacterium selectively enhanced. Terminal restriction fragment length polymorphism (T-RFLP) analysis of 16S rDNA suggested that N-Serve® selectively increased *Pseudomonas putida* and reduced bacterial diversity 72 h after application. *In vitro* tests revealed that the observed population increases of fluorescent pseudomonads were preceded by an early growth suppression of indigenous aerobic heterotrophic bacteria (AHB) population. Interestingly, the fluorescent pseudomonad population did not undergo this decrease, as shown in competition assays. Xylene and 1,2,4-trimethylbenzene (i.e., the inert ingredients in N-Serve®) were responsible for a significant percentage of the fluorescent pseudomonad population increase. Furthermore, those increases were significantly higher when the active ingredient (i.e., nitrapyrin) and the inert ingredients were combined, which suggests a synergistic response. This study demonstrates the possibility of using a specific substrate to selectively enhance and maintain desired populations of a natural-occurring bacterium such as *Pseudomonas putida*, a trait considered to have great potential in biocontrol applications for plant protection.

INTRODUCTION

Greenhouse growers are facing strict governmental regulations concerning the discharge of spent nutrient solution in order to abate ground water pollution resulting from nutrient salts and agricultural chemicals. Additionally, the use of recaptured irrigation water by the agricultural/horticultural industry is increasing due to pressure by governmental agencies to reduce water usage. Although the use of recycled irrigation water will reduce water usage and mitigate nutrient runoff from nursery production sites, serious grower concerns exist regarding the spread of phytopathogenic microorganisms via the recycled water. Management of these phytopathogens has been attempted using various biological, cultural, chemical, and physical methods [32, 40; 45, 49]. While these methods provide a limited measure of control for specific pathogens, the search for new methods or approaches is continuing.

During the course of our investigations on the control of zoosporic pathogens in hydroponic cultural systems, we discovered that amending the recirculating nutrient solution with N-Serve®, (a chemical which selectively inhibits denitrifying bacteria, i.e., *Nitrosomonas* spp., Dow AgroSciences) resulted in suppression of root rot of pepper caused by *Phytophthora capsici* [Pagliaccia *et al*., (2005) Abstract Phytopathology 95]. Additionally, preliminary studies indicated that the population of fluorescent pseudomonads appeared to be higher in N-Serve®-amended compared to non-amended treatments [Pagliaccia *et al*., (2004) Abstract Phytopathology 94]. Several fluorescent pseudomonads have been associated, via diverse mechanisms, with the suppression of root diseases caused by numerous fungal and fungal-like plant pathogens [14, 32, 44, 52]. Additionally, some isolates are recognized as plant growth promoters (PGP), and biopesticide/pollutant degraders [3, 19, 38; 48, 51].

The commercial product of N-Serve®24 contains 75.55% of inert ingredients (Primary inert ingredients: 23% 1,2,4-trimethylbenzene and 2% of xylene-mixed isomers), and 22.2% of active ingredient nitrapyrin [2-chloro-6-(trichloromethyl) pyridine], plus 2.25% related chlorinated pyridines. With respect to the role N-Serve® on the bacterial population, the degradation and utilization of solvents (i.e. inert ingredients) such as xylene and 1,2,4-trimethylbenzene, as carbon sources by pseudomonads, has been reported [42]. No information is available on the effect of nitrapyrin on the general bacterial population and, more specifically on the pseudomonad population. Nitrapyrin is a pyridine, known to

have a very selective effect on *Nitrosomonas* by chelating the copper components of enzymes involved in ammonia oxidation [35]; but, it also has some bactericidal effect, i.e. *Nitrosomonas* are not only temporarily inhibited in their activity, but part of the population is killed in treated soil [47]. Therefore the role of nitrapyrin as antimicrobial and metal chelator appears promising, especially in a hydroponic system scenario.

Biochelators, with different functions, are also produced by microrganisms. Biochelators, such as the siderophore pyoverdine, have specific roles in iron acquisition and transport by microorganisms [41] while others are responsible for the transport of some other metals. Beside pyoverdine, microorganisms produce a variety of biochelators with secondary functions such as redox activity, or chemicals with antibiotic activity, or induction of resistance against pathogens in plants [7, 29]. Among them, of particular interest, is the pyridine-based siderophores PDTC [pyridine-2,6-bis(thiocarboxylic acid)], a small metabolite secreted by certain pseudomonads (*P. stutzeri* and *P. putida*) when grown in iron-limited conditions [21, 22, 25]. PDTC has a remarkably strong affinity for various metals, antimicrobial activity toward several species of bacteria and, more interestingly, like the pyridine nitrapyrin (N-Serve® active ingredient), also undergoes hydrolysis forming similar by-products: PDTC forms dipicolinic acid (dpa) and nitrapyrin is rapidly degraded into 6-chloropicolinic acid [8, 50].

Encouraged by preliminary results, and aware of the fact that PDTC has analogy with nitrapyrin, a series of experiments were conducted to determine the effects of N-Serve® on bacterial population dynamics in amended versus non-amended hydroponic cultural systems, and the role of its active ingredient and the ‘inert components’ on the fluorescent pseudomonad populations.

MATERIALS AND METHODS

Greenhouse studies

Hydroponic cultural systems. Experiments were conducted in closed recirculating hydroponic cultural systems in temperature-controlled greenhouses (20-32°C) (Fig. 1.1). Two different host plants, gerbera (*Gerbera jasemonii* cv. Timo) and pepper (*Capsicum annuum* cv. Joe Parker) were used in order to assess the influence of different plants species on the response of bacteria following chemical amendments to the nutrient solution. A commercial nitrification inhibitor, (N-Serve® 24, Dow AgroSciences, Indianapolis) was used as the amendment to the recirculating nutrient solution in greenhouse experiments. The commercial product contains 22.2% nitrapyrin, 2.2% related chlorinated pyridines, and 75.55% inert ingredients (xylene and 1,2,4-trimethylbenzene are the primary inert ingredients).

Gerbera. The hydroponic recirculating system used in the gerbera greenhouse experiment was designed as previously described [13] with some modifications to allow randomization of the plant-growing containers on two greenhouse benches and to adapt the units to a recirculating drip system rather than a continuous flow system. Two individual recirculating units were used. Each unit consisted of six, 21-liter tub, a 30-liter reservoir and a 50-liter refill tank. Three black polyethylene pots (24.5 cm x 10.2 cm x 10.16 cm), with one plant in each, were placed in each of the six, 21-liter tubs. Pots tapered downward to allow free drainage of nutrient solution. The nutrient solution was pumped out from the reservoir through of a network drip irrigation system into each individual pot. Irrigation time and intervals were controlled by a timer to provide for 5-minute watering duration every two hours between 8.00 am and 8.00 pm. The nutrient solution contained the following nutrients: 16 NH_4NO_3, 482.23 KNO_3, 708.45 $Ca(NO_3)2x4H_2O$, 52.82 $(NH_4)_2HPO_4$, 81.6 KH_2PO_4, 1.73 H_3BO_3, 283.45 $MgSO_4x7H_2O$, 0.049 $CuSO_4x5H_2O$, 3.48 K_2SO_4, 1.72 $ZnSO_4xH_2O$, 0.61 $(NH_4)_6Mo_7O_{24}x4H_2O$, 0.79 $MnCl_2$, 28.93 Fe EDTA (98%) $\mu g\ ml^{-1}$. Electrical conductivity was measured weekly using a Horiba B-173 conductivity meter (Spectrum Technologies, Inc., Plainfield, IL). Solution pH was adjusted manually to 6.0 ± 0.2 with H_3PO_4 or KOH every week. Nutrient solution temperatures were monitored every 30 minutes, using a temperature data logger (HOBO, ONSET Computer Corporation).

Gerbera plant (*Gerbera jasemonii*), cv. Timo, at the third-leaf stage was transplanted into black polyethylene pot (24.5 cm x 10.2 cm x 10.16 cm), filled with perlite. One month after transplant, the nutrient solution in each unit was replaced with fresh nutrient solution. There were two gerbera experiments and each consisted of two treatments: N-Serve®-amended and non-amended nutrient solution. N-Serve®, at 25 µg a.i. ml^{-1}, was applied four times at three weeks intervals. Each treatment included 18 plants.

Pepper. Experiments using pepper as the host were conducted in a temperature-controlled (24 to 34°C) greenhouse using 4 separate recirculating hydroponic units with top irrigation system. Each hydroponic unit consisted of a plastic trough, located on a bench ca. 40 cm above ground level and connected to a 50 l-nutrient solution reservoir. Each trough contained 6 potted plants. The nutrient solution was pumped from the reservoir to the bench top and distributed to each plant via drip tubing with 7.5 l/h emitters. Excess nutrient solution from each pot drained by gravity back to the reservoir and was recirculated. Plants were irrigated for five minutes every two hours between 8:00 am until 8:00 pm. The solution pH was adjusted manually to 6±0.2 with H_3PO_4 and KOH, every week. The electrical conductivity (EC 2±0.2 mS/cm^2 for pepper) and the nutrient solution volume in reservoirs (25 l) were monitored twice a week and levels maintained as required. The final elements concentrations in the nutrient solution were as follows: Mg 48.8, S 64.5, P 62.1, K 241.5, N 222.7, Ca 235.1, B 0.44, Cu 0.05, Cl 0.85, Mn 0.62, Mo 0.06, Zn 0.09, and Fe 2.5 µg ml^{-1}.

Three-week-old pepper seedlings grown in Oasis Horticubes (Smithers-Oasis, Kent, OH) in a temperature-controlled incubator were transplanted to 11 cm × 11 cm × 12 cm plastic pots containing a commercial organic (peat-based) potting mix (Supersoil®, Rod McLellan Company, San Mateo, CA). Potted pepper plants were then placed in troughs and grown for 4 days before treatment. There were two experiments each consisted of two treatments with 6 plants per treatment: N-Serve®-amended and non-amended nutrient solution. N-Serve®, at 25 µg a.i. ml^{-1}, was first applied 5 days after the start of the experiment and reapplied at day 12, 26 and 33. In all greenhouse experiments, N-Serve® was added to the nutrient solution 30 minutes before the start of the irrigation.

Monitoring bacterial populations in nutrient solutions. Twenty ml samples of the nutrient solution were collected at different interval (see Fig. 1.3 and Fig. 1.4) from the

reservoir of each treatment. After serial 10-fold dilutions, aliquots were plated in triplicates, using a spiral plater (Autoplate 4000 – Spiral Biotech, Inc) onto 10 cm diameter petri dishes containing King's B medium (KB) for enumeration of the fluorescent pseudomonads and Tryptic Soy Agar (TSA) for enumeration of the aerobic heterotrophic bacteria. TSA was supplemented with 100 μg ml^{-1} cycloheximide (Calbiochen) and KB agar was supplemented with 50 μg ml^{-1} cycloheximide and 75 μg ml^{-1} penicillin (Calbiochen). Colonies were counted after 24 hours incubation at 28° C. Fluorescent pseudomonads were counted under UV light.

Bacterial collection and identification. Eighty-five colonies of predominant bacteria growing on TSA medium were collected from the N-Serve®-amended (50 isolates) and non-amended (35 isolates) nutrient solutions from the gerbera and pepper experiments. Petri dishes containing 50–100 single colonies were chosen and colonies were randomly selected from a different sector of each plate. The isolated colonies were purified by repeated streaking onto King B agar. Purified isolates were stored at room temperature on KB agar plates or maintained for long-term storage at –70 °C in nutrient broth (Difco Laboratories) supplemented with 15% glycerol. Eighty-five bacteria isolates were identified using the BIOLOG™ GN plates and the Biolog Microlog2 4.20 software database (Biolog, Inc, Hayward, CA).

Genomic DNA extraction. Samples of the gerbera nutrient solution from the reservoirs (1 L of hydroponic nutrient solution) from each treatment were collected onto sterile nitrocellulose membranes of two different sizes, 0.22 and 8 μm (Millipore, nitrocellulose) using a sterile vacuum aspirator filtering apparatus. The filter-concentrated bacterial cells were re-suspended in 6 ml buffer 50 mM Tris-HCl pH 9, 20 mM EDTA, 400 mM NaCl, 0.75 M sucrose and stored until extraction procedure in –80°C freezer. Genomic DNA was extracted from bacterial suspension and pure cultures using the DNeasy Tissue Kit (Qiagen, Hilden, Germany). The purity and the concentration of the DNA template was verified by electrophoresis in a 1% agarose gel and stained in ethidium bromide solution.

PCR amplification and T-RFLP analysis. Bacterial 16S rRNA genes were amplified by polymerase chain reaction (PCR) with the primers FAM-63F, 5'-CAGGCCTAACACATGCAAGTC-3' labelled at the 5' end with 6-carboxyfluorescein

dye, and 1389R, 5'-ACGGGCGGTGTGTACAAG-3' [30] following the thermal profile described by Bertin *et al*., [2]. Fluorescently labelled PCR products were purified with QIAquick PCR purification kit (Qiagen) and 100 ng were digested with 10 U of the restriction enzymes RsaI or HhaI (Invitrogen, Italia) for at least 4 h at 37°C. The digested products (2 µl) were mixed with 19.5 µl of deionised formamide and 0.5 µl of ROX-labeled GS500 internal size standard (Applied Biosystems) and denaturated for 5 min at 95°C before capillary electrophoresis on ABI Prism 310 Genetic Analyzer (Applied Biosystems). Electropherogram analysis was performed using GeneScan Analysis 3.1 software using the local southern size calling method. The computational method developed by Abdo *et al*. [1] was used to determine a baseline, for indentification of the "true" peaks in electropherograms, and to compare electropherograms binning fragments of similar size.

In order to investigate the distribution of bacterial diversity in the analysed samples, the peak relative intensity was statistically elaborated. Pairwise similarity between whole communities were analysed by calculating Jaccard coefficient ($S_j = W/(a_1 + a_2 - W)$) and Whittaker index of association ($S_w = 1-\sum |b_{i1}-b_{i2}|/2$), where W is the number of peaks shared between population 1 and 2; a_1 and a_2 are the total number of different peaks in population 1 and 2; b_{i1} and b_{i2} are the relative fluorescence of each ith peak in samples 1 and 2 [23]. The Shannon-Weaver index ($H = -\sum P_i \log P_i$) and Simpson index ($1-D = 1-\sum P_i^2$) were determined according the description given in [23], where P_i is the relative fluorescence of each ith peak. The values of ecological indices obtained for amended and non amended sample were compared by using Student's *t* test.

DNA sequencing and phylogenetic analysis. 16S rRNA genes were amplified as described before from genomic DNA samples obtained from pure cultures of the strains isolated from the hydroponic nutrient solution. Unlabeled PCR products, purified as described above, were cloned using the pGEM-T Easy Vector System (Promega) according to the manufacturer's instructions and plasmid DNA, purified using a Wizard Plus SV Mini Prep kit (Promega), was subjected to cycle sequencing using the M13 primers and the BigDye Terminator Cycle Sequencing Ready Reaction kit (Applied Biosystems). The DNA sequences were bi-directionally resolved on an ABI Prism 310 in a

sequencing mode. Nucleotide sequences were assembled and compared with the sequences in the RDP database (Ribosomal Database Project) to identify the closest relatives.

The 16S rRNA gene sequences obtained in this study are available from the EMBL nucleotide sequences database under Accession number AM259176, AM259177, AM259178.

In vitro substrates utilization

Bacterial strains used for in vitro studies. The following bacterial strains were isolated from the hydroponic nutrient solution: isolate 32, identified using BIOLOG^{TM} system as *Aeromonas hydrophila*, isolate 34, identified as *Aeromonas caviae*, isolate 36 and isolate Gerr identified as *Pseudomonas putida* by nucleotide sequencing of 16S ribosomal DNA. Inocula for all *in vitro* experiments were prepared by growing the isolates first in LB broth (EMD Chemicals Inc.) at 28°C in a shaker (120 rpm) for 24 h, then in KB agar for 48 h. Finally loops were transferred into 10 ml of 0.01 M $MgSO_4$ solution. These cells were washed twice in $MgSO_4$ and CFU ml^{-1} assessed at 600 nm using Genesys 10vs Spectrophotometer (Spectronic Unicam). These suspensions were then diluted to desired population densities for in vitro studies.

Effect of N-Serve® on the indigenous bacterial populations. Four, 0.5-liter samples of gerbera nutrient solution were collected from a non-treated reservoir in the greenhouse and then amended in the laboratory with 0, 25, 50 or 100 µg a.i. ml^{-1} N-Serve®. After 30 minutes, aliquots were plated in triplicates on KB agar and TSA agar then incubated at 28° C for 24 h and bacterial counts recorded as described above.

Effect of the inert and active ingredients of N-Serve® on the indigenous fluorescent pseudomonads. The active ingredient (i.e., nitrapyrin) and inert ingredients (i.e., xylene and 1,2,4-trimethylbenzene) of N-Serve®, either alone or in combination, were used as amendments to assess their effect on the bacterial population in the nutrient solution.

The nutrient solution with no chemical amendments served as the control. Nitrapyrin (90%, N-ServeTG®) at 12.5 µg a.i. ml^{-1} and/or xylene (99.7%, Mallinkrodt Baker, Inc.) at 20 µg ml^{-1} mixed with 1,2,4-trimethylbenzene (98%, Sigma-Aldrich, Inc) at 20 µg ml^{-1} were added to 0.5L of non-sterile nutrient solution collected from the hydroponic pepper

recirculating system and incubated at 28 °C with continuous agitation on a rotary shaker (100 rpm). Fluorescent pseudomonad population in the chemically-amended nutrient solutions were monitored daily for 6 days on King's B agar as described above. The concentrations of the inert ingredients used in the above experiments approximated those in the commercial formulation of N-Serve®.

Effect of N-Serve® on Pseudomonas putida Gerr in sterile nutrient solution and sterile distilled water. To study the effect of N-Serve® on the growth of *P. putida* Gerr (a member of *P. putida* population selectively enhanced in gerbera experiment) in the absence of competition from other bacteria, the bacterium was added to sterile nutrient solution and sterile distilled water. Nutrient solution was collected from a non-treated reservoir of a hydroponic pepper experiment and filter sterilized by passing through 8 and 0.22 μm filters (Millipore, nitrocellulose). N-Serve® (25 μg a.i. ml^{-1}) was added to 0.5L of both the sterile water and sterile nutrient solution and seeded with 4.4 log CFU ml^{-1} of *P. putida*. The treatments were incubated at 28 °C with continuous agitation on a rotary shaker (100 rpm). Bacterial populations were measured daily by plating serial dilutions onto King's B agar with a spiral plater (Autoplate 4000 – Spiral Biotech, Inc.). The plates were incubated at 28 °C and after 48 h colonies were counted under UV light.

Growth of three indigenous bacterial strains, introduced alone or in mixture, in sterile nutrient solution amended with N-Serve®. Three dominant and indigenous isolates of bacteria recovered from the nutrient solution [(i.e, *Aeromonas hydrophila* (isolate 32), *Aeromonas caviae* (isolate 34) and an unidentified fluorescent pseudomonad *(isolate 36*)] were individually assessed for their ability to grow in sterile nutrient solution amended with and without N-Serve® at 25 μg a.i. ml^{-1}. The nutrient solution for these experiments was collected from a hydroponic pepper experiment, and filter sterilized as described above. Inocula were prepared as previously described and then added individually, in triplicates, to 200 ml of sterile pepper solution to give a final concentration of ca. 3.1 (log CFU ml^{-1}). After 24 h bacterial populations were enumerated as described previously.

To investigate the behavior of the isolates in a mixture, *A. hydrophila, A. caviae* and the fluorescent pseudomonad, were each added at ca. 3.7 log CFU ml^{-1} to 200 ml of sterile

pepper solution amended with N-Serve® (25 µg a.i. ml^{-1}). The same concentration of this mixture, add to sterile nutrient solution served as a control. After 24h, bacterial populations in the various treatments were enumerated as described previously.

Data analyses. All data were log_{10} transformed before statistical analysis. Data on the bactericidal effect of N-Serve® on indigenous pseudomonads and AHB populations (Fig. 1.6) were analyzed by the one-way repeated measures analysis of variance, followed by the all Pairwise Multiple Comparison Procedures (Holm-Sidak method). *In vitro* studies on the growth stimulatory effects of the active versus inert ingredients of N-Serve® (Fig. 1.7) on indigenous bacteria, 72 hours after treatment, were analyzed by one-way analysis of variance followed by the all Pairwise Multiple Comparison Procedures (Holm-Sidak method). Data on the individual bacterial ability to grow on N-Serve® and the data on the competition assays (Table 1.3 and Table 1.4) were analyzed by three-way analysis of variance followed by the Student-Newman-Keuls pairwise multiple comparison procedures.

The observed effects were considered statistically significant when the calculated P-value is below 0.001. All data were analyzed using SigmaStat 3.0 statistical software package (SPSS Science, Chicago, IL). All experiments were repeated at least once and results from a single representative experiment were chosen for presentation.

RESULTS

Greenhouse studies

Monitoring bacterial populations in the nutrient solutions. Prior to the addition of N-Serve®, the recirculating nutrient solution used to irrigated gerbera plants contained ca. 3.6 log CFU ml^{-1} of aerobic heterotrophic bacteria (AHB) (Fig. 1.3A) and an undetectable level of fluorescent pseudomonads (Fig. 1.3B). However, within 72 hrs after the addition of N-Serve®, the average population of fluorescent pseudomonads in amended treatments (ca. 6 log CFU ml^{-1}) were 2-3 orders of magnitude greater than that in non-amended treatments (ca. 3 log CFU ml^{-1}) (Fig. 1.2 and 1.3B). The population surge occurred after each consecutive addition of N-Serve® to the nutrient solution. The average fluorescent pseudomonad population accounted for ca. 80% (ranging from 65 to 100%) of the total

AHB population in the N-Serve®-amended solutions but only 2% (range from 0.3 to 4.3%) in the non-amended nutrient solution. Similar significant increases in the resident fluorescent pseudomonad population, compared to the non-amended control treatment, were also consistently recorded in the pepper experiments following the addition of N-Serve® to the recirculating nutrient solution (Fig. 1.4).

Bacterial identification. Among the 50 isolates of dominant bacteria, randomly selected (on TSA medium) from N-Serve®-amended nutrient solutions, 94% were fluorescent pseudomonads. Forty-five isolates (90%) were identified using Biolog™, with similarity indices of 75% (ranging from 70-91%), as *Pseudomonas putida* biotype A, and two (4%) as *Pseudomonas putida* biotype B. Three nonfluorescent isolates (6%) were identified with Biolog™ as *Stenotrophomonas maltophilia.* Moreover, 16S rDNA sequences of eight selected isolates were analysed and identified as *P. putida* (4 isolates), *P. fluorescens* (2 isolates) and *S. maltophila* (2 isolates). Comparative analysis of these sequences with the RDP database showed significant similarities with 16S rRNA gene sequences of plant commensal *Pseudomonas*, bacterial strains with antifungal, antioomycete and algicidae activities, Polycyclic Aromatic Hydrocarbons (PAHs) and pesticides degrading bacteria, environmental and clinical isolates of *Pseudomonas*; S_{ab} values (similarity coefficient for query and matching sequences) were between 0.85 than 0.99.

In contrast, of the 35 randomly selected isolates (on TSA medium) of dominant bacteria, recovered from the non-amended nutrient solution, none were identified as *Pseudomonas putida*. Fifty-seven percent (20 isolates) could not be identified to species using Biolog™ since the similarity indices were below 50%. The remaining 43% (15 isolates) of dominant isolates were identified, with similarity indices of 67% (ranging from 53 to 91%), as *Pseudomonas synxantha* (5 isolates), *Pseudomonas fulva* (3 isolates), *Pseudomonas fluorescens biotype C* (1 isolate) *Aeromonas caviae* DNA group 4 (2 isolates), *Aeromonas hydrophila* DNA group 1 (2 isolates), *Delfia acidovorans* (1 isolate) and *Comamonas testosteroni* (1 isolate).

Community structure analysis. The distribution and the structure of the bacterial community in response to the addition to N-Serve® to the recirculating nutrient solution were evaluated using a molecular approach. The analysis of terminal restriction fragment length polymorphism (T-RFLP) of 16S rRNA genes has proven to provide a culture independent method to compare microbial communities and presumptively identify abundant members. Fluorescently labeled universal primers that anneal to conserved region of 16S rRNA genes in *Bacteria* (see Experimental procedures) gave amplification products of the expected size in all the samples analyzed. Data from T-RFs patterns generated with *Rsa*I or *Hha*I as restriction enzymes were combined to achieve a more accurate characterization of the microbial communities in the nutrient solutions. As illustrated in Fig. 1.5, differences in profiles, as well as changes in numbers of discernible peaks, could be seen among the samples from solution amended or not with N-Serve®. To identify "true" peaks and compare electropherograms binning fragments of similar size, the method developed by Abdo *et al*. [1] for the analysis of T-RFLP data was used. The results obtained were shown in Fig. 1.3C where the data were normalized by calculating the relative peak areas. *Rsa*I and *Hha*I digestion of fragments amplified with universal primers for *Bacteria* generated 12 peaks. The predominant *Rsa*I T-RFs in control samples from untreated nutrient solution were sized 383 bp, and in profiles obtained with *Hha*I digestion the major peak had a size of 79 bp. The T-RFs pattern of samples amended with N-Serve® was less complex than the one generated with non amended samples and showed the presence of a peak that alone account for more than 59% of the total peak area of the sample (608 bp *Rsa*I profiles, 170 bp *Hha*I profiles).

The whole profile analysis of T-RFLP data showed that the fragments that were represented in T-RFs profiles of samples amended with N-Serve® were also present in non amended samples, but the relative abundance of each phylotype was significantly different. The Jaccard coefficient, which indicates the similarities between T-RFs patterns considering the presence/absence of the bands, had a value of 0.42 (*Rsa*I) and 0.58 *(Hha*I); the Whittaker coefficient, which takes into consideration the relative intensity of each band, had lower values (0.34 and 0.42 respectively), supporting the hypothesis that the structure of the bacterial communities was affected by the addition of N-Serve®.

In order to describe the changes in the dominance among the phylotypes, the ecological diversity indices of Shannon-Weaver and Simpson were calculated (Table 1.1).

Diversity indices for both enzymes were not significantly different (P>0.13), indicating that the diversity of the samples was independent from the restriction enzyme used to generate the T-RFs profiles. On the contrary, indices in samples amended with N-Serve® were significantly lower than those obtained in non amended samples (P<0.01). These data suggest that the addition of N-Serve® decreases the prokaryotic diversity in the sample, thus reducing the species richness and evenness and enhancing a single phylotype of the bacterial consortium.

T-RFLP analysis, performed on pure culture DNA of Gerr isolate generated a single T-RF of 170 bp after *Hha*I digestion and 608 bp fragment after *Rsa*I digestion (data not shown), indicating that the dominant fluorescent specie in the nutrient solution samples amended with N-Serve® matched with the *P. putida* isolate.

In vitro substrates utilization.

Effect of N-Serve® on the indigenous bacterial populations. The population densities of resident AHB decreased significantly (50-85%) (Fig. 1.6A), compared to those in the non-amended nutrient solution, within 30 minutes after the addition of 25, 50 and 100 µg a.i. $ml^{-1}$of N-Serve® to the nonsterile nutrient solution. In contrast, no significant reduction in population density of indigenous fluorescent pseudomonads (IFP) (Fig. 1.6B) was observed in the nonsterile nutrient solution amended with 25 and 50 µg a.i. ml^{-1} of N-Serve®. However, a significant decrease (40%) in the IFP population was observed in the nutrient solution amended with 100 µg a.i. $ml^{-1}$of N-Serve®.

Effect of the inert and active ingredients of N-Serve® on the indigenous fluorescent pseudomonads. Seventy-two h following the addition of the inert ingredients (xylene and 1,2,4-trimethylbenzene) or a mixture of the active ingredient (nitrapyrin) plus the inert ingredients (xylene and 1,2,4-trimethylbenzene) to the non sterile nutrient solution in the laboratory, the mean population densities of indigenous fluorescent pseudomonads increased from 2.7 to 4.6 log CFU ml^{-1} and from 2.70 to 6.13 log CFU ml^{-1}, respectively (Fig. 1.7). Significant increases in population densities in the mixture treated solution, was observed compared to the inert ingredients amendment. In contrast, the population density of indigenous fluorescent pseudomonads decreased during the same time frame in both the unamended and nitrapyrin-amended treatments. The drop in population densities was not

considerably different (although with a significant greater survival with nitrapyrin after 72 hour) but both were significantly different from the nitrapyrin plus inert ingredient and inert ingredient treatments.

Effect of N-Serve® on Pseudomonas putida Gerr in sterile nutrient solution and sterile distilled water. Within 72 h following the addition of N-Serve® to sterile nutrient solution seeded with *P. putida* Gerr (see experimental procedures), bacterial counts increased from 4.40 to 5.98 log CFU ml^{-1}but decreased rapidly in the absence of N-Serve® (Table 1.2). In contrast, bacterial counts decreased from 4.4 log CFU ml^{-1} to non-detectable levels within 24 h following the addition of N-Serve® to sterile distilled water. A decrease in the population of *P. putida* Gerr also occurred in the non-amended sterile water but the decrease was less than in the N-Serve®-amended treatment.

Growth of three indigenous bacterial isolates, introduced alone or in mixture, in sterile nutrient solution amended with N-Serve®. Significant increases in the populations of each of three (i.e., two nonfluorescent and one fluorescent) members of the dominant indigenous bacterial isolates (which were isolated from unamended nutrient solution) occurred in sterile nutrient solution amended with N-Serve® (Table 1.3). No increase occurred in the absence of N-Serve®. Additionally, a significant increase in the population, relative to the control, was also observed following the addition of all three bacteria as a mixture to sterile nutrient solution amended with N-Serve®. However, the fluorescent pseudomonad accounted for almost 100% of the observed population increase (Table 1.4). Comparison analysis confirmed that there was no significant difference (P = 0.561) between total bacteria population and fluorescent *Pseudomonas* isolate 36 population, 24 hours after N-Serve® amendment.

DISCUSSION

Fluorescent pseudomonads have historically been associated with suppression of root diseases caused by numerous fungal and fungal-like pathogens. However, inconsistency in performance has been a problem. This has been attributed, in part, to the inability of the biocontrol bacteria to maintain critical threshold populations necessary for sustained biocontrol activity. It is becoming increasingly evident that biocontrol bacteria will not be effective for sustained period of time unless the environment is modified to make it more conducive for their growth and survival [5].

Our results indicate that a nitrogen stabilizer (N-Serve®, Dow Agrosciences) selectively and significantly enhanced the fluorescent pseudomonad population in the recirculating nutrient solution reservoirs in both hydroponically-grown gerbera and pepper plants (Fig. 1.3 and Fig. 1.4). Our results support and extend the concept of selective enhancement of a specific microorganism in a root/soil microbial community by providing an exotic substrate. The use of nutritional amendments has previously been reported to enhance the efficacy of several bacterial and fungal biological control agents of fungal diseases [15]. For example, Colbert *et al.,* [4, 5, 6] demonstrated that amending soil with salicylate selectively increased the metabolic activity and populations of an introduced strain (PpG7) of *Pseudomonas putida.* Yamada [55] demonstrated that amending soil with methionine enhanced the population of *Pseudomonas putida* AP-1 which increased suppression of *Fusarium* wilt of tomato. Similarly, olive oil, when added to the recirculating nutrient solution of hydroponically grown peppers, stimulated the metabolic activity (i.e., rhamnolipid production) and population density of an introduced strain (R4) of *P. aeruginosa* which resulted in the control of *Phytophthora capsici* [44]. Utilization of exotic substrates to enhance specific microorganisms is not restricted to root/soil microcosms but has also been reported for foliar microcosms [9, 16, 35, 53-55].

In contrast to the above studies in which the microorganism intended for enhancement were intentionally introduced into the desired microcosm, the bacterium enhanced in our studies existed naturally, at low population densities, in the nutrient solution. The enhanced bacterium was subsequently identified, using BIOLOG™ identification test and comparison of 16S rRNA gene sequences (Fig. 1.5), as a *Pseudomonas putida* strain. This bacterium, either directly or indirectly, was associated, with disease suppression in our hydroponic cultural systems [Pagliaccia *et al*., (2005)

Abstract Phytopathology 95; 31]. A role in biological control by *Pseudomonas putida*, has been shown [14, 51].

Coupled with the enhancement of *Pseudomonas putida* population, a reduction in species richness (i.e., a lower bacterial diversity expressed as number of T-RFs), occurred in the N-Serve®-amended compared to the un-amended nutrient solution (Fig. 1.5A and 5B). It has been reported that diversity of microbial communities can be affected by organic pollutants and that organic chemicals reduce microbial diversity in different environments [11, 38]. Our *in vitro* study supports these conclusions. Specifically, we observed that the bacterial population bursts in our study was preceded (within 30 minutes after amending the nutrient solution with N-Serve®) by early growth suppression of the indigenous AHB population while no effect was observed on the fluorescent pseudomonad population (Fig. 1.6). After this lag period, the fluorescent pseudomonad population was selectively enhanced, as evidenced by data from the greenhouse (Fig. 1.3 and Fig. 1.4). These results are consistent with a previous report from Liu and Suflita [26] which observed that the composition of the indigenous microbial population in the soil and ground water will adapt to the presence of pollutants mainly because bacteria able to use these compounds substrates as a nutrient source would be able to proliferate and became dominant. In support of this hypothesis, our competition assays (Table 1.4) provided evidence that indigenous N-Serve®-tolerant bacteria out-compete others for available resources and the non-pseudomonad bacteria were the most impaired.

Thus, given that N-Serve® contains inert ingredients such as xylene and 1,2,4-trimethylbenzene, we hypothesized that the increase in fluorescent pseudomonad population could be the result of the degradation of those inert ingredients and their utilization as a carbon source. Our *in vitro* studies on the growth stimulatory effects of the active versus inert ingredients of N-Serve® (Fig. 1.5) on indigenous bacteria in the nutrient solution confirmed that the inert components of N-Serve®, used as carbon source, were responsible of a significant percentage of the bacterial population increase, and more specifically, the fluorescent pseudomonad population. These results are consistent with previous reports that pseudomonads are metabolically versatile and capable of utilizing many natural and xenobiotic compounds including xylene and benzene [12, 28].

Unclear and more ambiguous is the role of nitrapyrin on the bacterial growth stimulatory effect. Our laboratory studies showed that nitrapyrin, in contrast to the inert

ingredients, cannot be used by the bacteria as a sole carbon source, however prevented the rapid decline of the pseudomonad populations over time (Fig. 1.7). Furthermore, when the active and inert ingredients were mixed together, the increase of the bacterial population was significantly higher than those recorded in response to the inert ingredients alone (Fig. 1.7). In addition to the reduced competition due to the bactericidal activity of nitrapyrin (Fig. 1.6) and the use of the inert ingredients from pseudomonads as carbon source (Fig. 1.7) the metal chelator properties of nitrapyrin [35] may also play an important role in the bacterial growth stimulation. The formation of complexes between nutrient solution soluble metals and nitrapyrin could render essential metals for bacterial growth available only to pseudomonads which posses such metal acquisition systems. Thus pseudomonads gain a significant growth advantage over other bacterial species.

The selectively enhanced pseudomonads growth patterns described above occurred in experimental systems using recycled hydroponic nutrient solution which contained inorganic nutrients, organic and inorganic matter. When a pure culture of fluorescent *Pseudomonas putida* isolate Gerr grown in distilled water was treated with N-Serve® a rapid decrease in bacterial growth was observed. When the same strain was grown in a sterile hydroponic nutrient solution treated with N-Serve® the pseudomonas population densities increased by three log units (Table 1.2). It has been reported that pseudomonas have diverse metabolic pathways controlled by a variety of plasmid and chromosomal genetic mechanisms often affected by environmental factors [20, 33, 44]. More specifically Zhou and Crawford [55] reported that microbial degradation of organic compounds is strongly influenced by physical and chemical factors such as nutrients, temperature, oxygen, salinity, pressure, water activity, pH, and chemical composition. The importance of the inorganic nutrients (i.e. N and P) on the bacterial stimulation for pollutants degradation has also been reported [10, 42]. In our study, the nutrient-rich hydroponic solution, in combination with the stable pH, E.C. and oxygen supply most likely stimulated and sustained the expression of the catabolic mechanisms of the amended aromatic compounds resulting to the observed *Pseudomonas putida* enhanced populations. The results of the competitive assay with *A. hydrophila* and *A. caviae* against fluorescent pseudomonad further supported the metabolic durability and versatility of the florescent pseudomonads in the N-Serve amended hydroponic environment since the non

pseudomonas isolates were able to increase their population only in the absence of the florescent pseudomonads (Table 1.4).

Regardless of the specific mechanism controlling the selective population increase of fluorescent pseudomonads, since the population densities reached the 10^5-10^6 CFU ml^{-1} threshold [14, 31], one can only expect a proportionally increased production of the siderophores. The biocontrol activity of the fluorescent pseudomonads siderophores is well documented and has been demonstrated for several siderophores such as pyoverdines [24, 27, 36].

The significance of these studies could be of important value. First, it could solve one of the main problems associated with inconsistency in performance of a biocontrol agent, namely maintenance of a high population density of the selected biocontrol agent. The resident microflora in many, if not most, habitats could already contain prospective members that could function as antagonists to other microorganisms in the same habitat if selectively enhanced. This hypothesis is consistent with principles established for bioremediation by Kuiper *et al*. [17, 18], in which naturally-occurring bacteria were selected for the ability to both degrade a pollutant and colonize plant roots. In our case this principle could be used to insure the selection of a fluorescent pseudomonad population from the rhizosphere and the nutrient solution, for beneficial purpose such as biocontrol, biofertilization, and phytostimulation.

Second, N-Serve® was identified as a specific chemical which, when applied at low concentrations, can selectively enhance specific microbes amongst a milieu of others. More efficient compounds with similar properties could be identified or refined for their selective enhancement for specific biological purposes. While hydroponic systems with recirculating nutrient solutions offer optimal conditions for bacterial growth, i.e., the relatively high concentrations of root exudates, non-limiting minerals, high aeration and incubation temperatures of 20–30°C, the same principle may also have application in field agriculture. For example, these growth stimulating chemicals could be applied via the drip irrigation system and used to manipulate specific indigenous microbial populations for plant health management.

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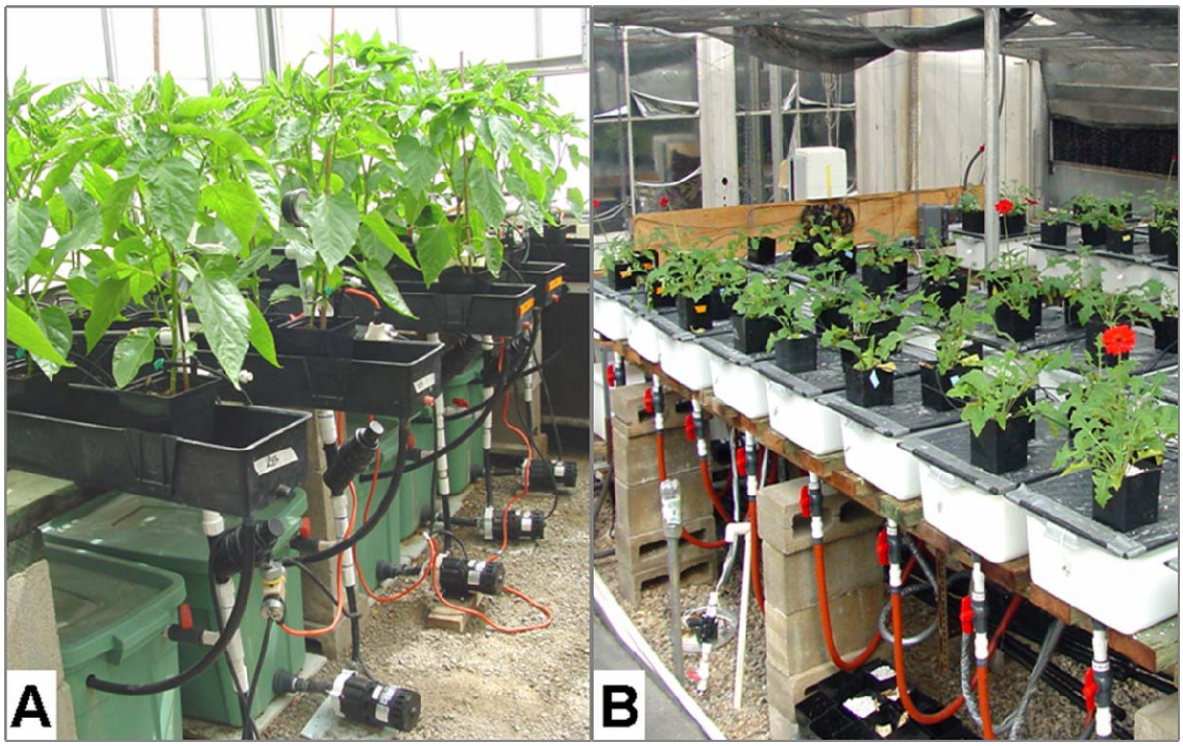

Figure 1.1. Pepper (A) and gerbera (B) plants in drip-irrigated hydroponic units in a temperature-controlled greenhouse.

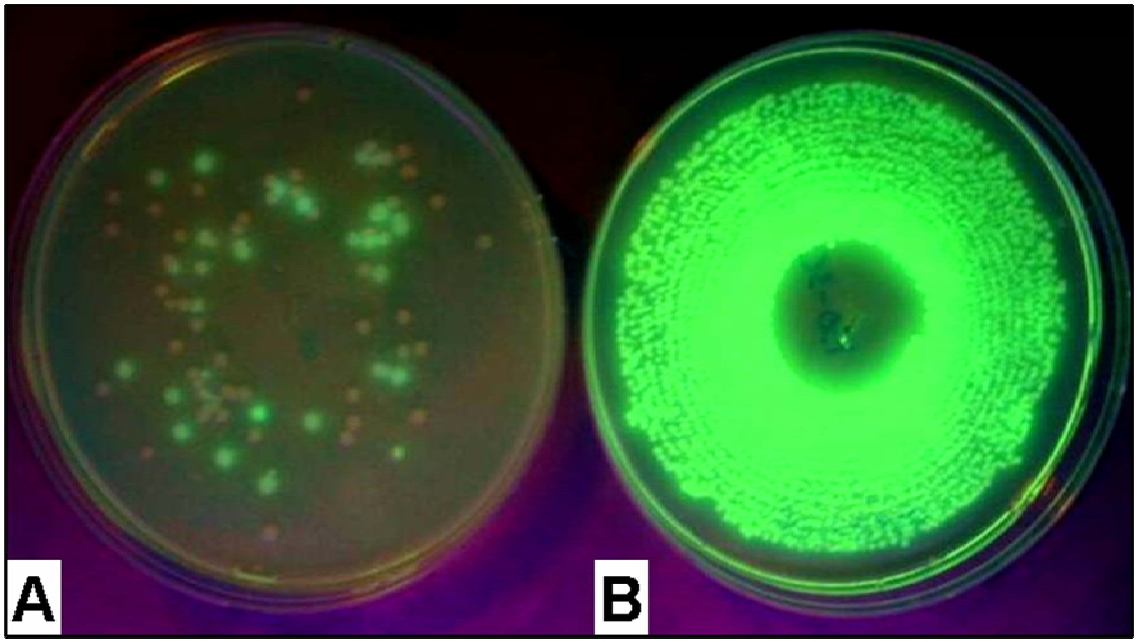

Figure 1.2. Fluorescent pseudomonad population in the recycled nutrient solution at time zero (10^3 CFU ml^{-1}) (A) and 72 h (10^6 CFU ml^{-1}) after N-Serve® treatment (B).

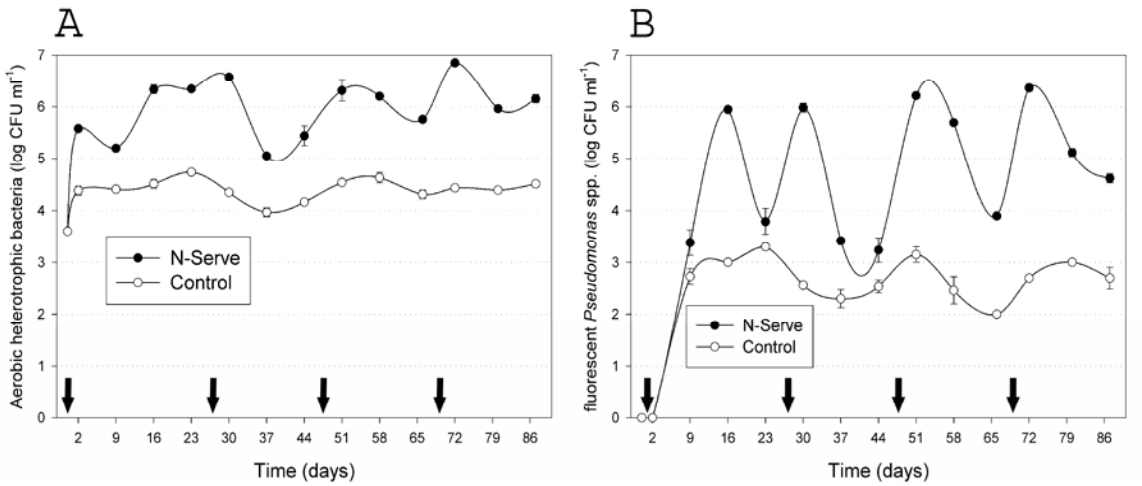

Figure 1.3. Mean population densities of aerobic heterotrophic bacteria (A) and fluorescent pseudomonads (B) in a greenhouse experiment with gerbera as the host plant. Treatments consisted of nutrient solution amended and not amended (control) with N-Serve® every 3 weeks. Arrows indicate the timing of N-Serve® applications (25 µg a.i. ml^{-1}). Error bars represent the standard error of the mean (n = 3) where variation was great enough to be presented.

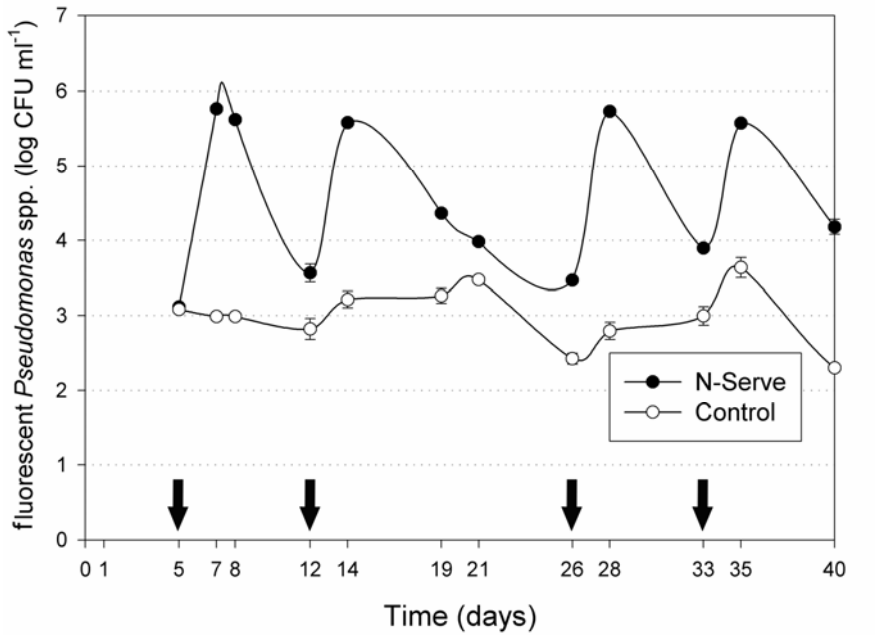

Figure 1.4. Mean population densities of fluorescent pseudomonads in a greenhouse experiment with pepper as the host plant. Treatments consisted of nutrient solution amended and not amended (control) with N-Serve®. Arrows indicate the timing of N-Serve® applications (25 µg a.i. ml^{-1}). Error bars represent the standard error of the mean (n = 3) where variation was great enough to be presented.

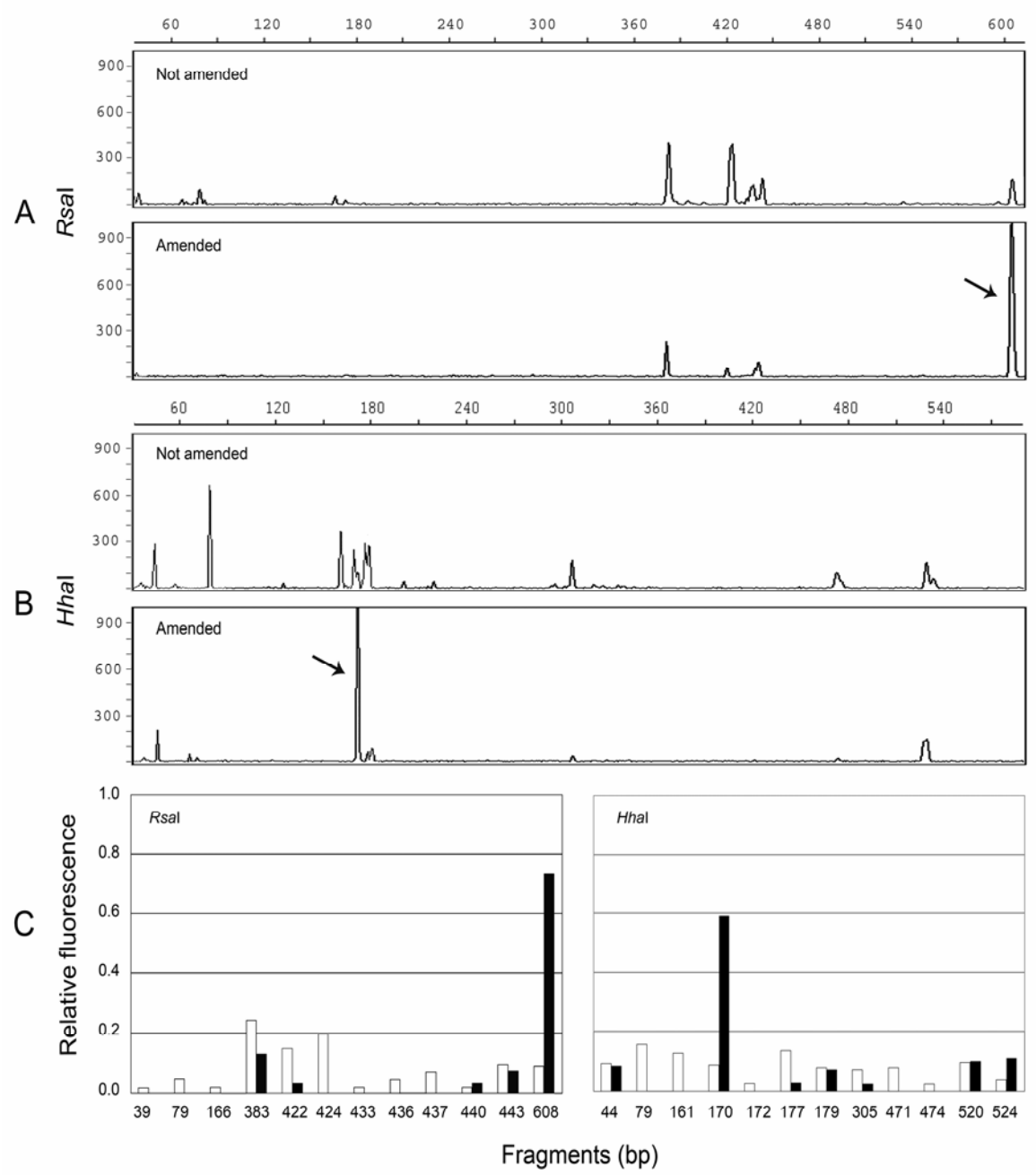

Figure 1.5. T-RFLP profiles from *RsaI* (A) and HhaI (B) analysis of 16S rDNA gene PCR products amplified from DNA isolated from amended and not amended gerbera nutrient solution, 3 days after N-Serve® (25 µg a.i. ml^{-1}) addition. Comparison of the relative abundance of T-RFs after *Rsa*I and *Hha*I digestion between not amended (white) and amended samples (black) (C).

Table 1.1. Values for ecological diversity indices of Shannon-Weaver (H) and Simpson (1-D) obtained by using the data of the *Hha*I and *Rsa*I generated T-RFLP.

	*Hha*I T-RFs pattern		*Rsa*I T-RFs pattern	
	H	1-D	H	1-D
Not amended	2.36 ± 0.21	0.90 ± 0.07	2.15 ± 0.13	0.85 ± 0.09
Amended	1.36 ± 0.08	0.62 ± 0.05	0.91 ± 0.10	0.44 ± 0.03

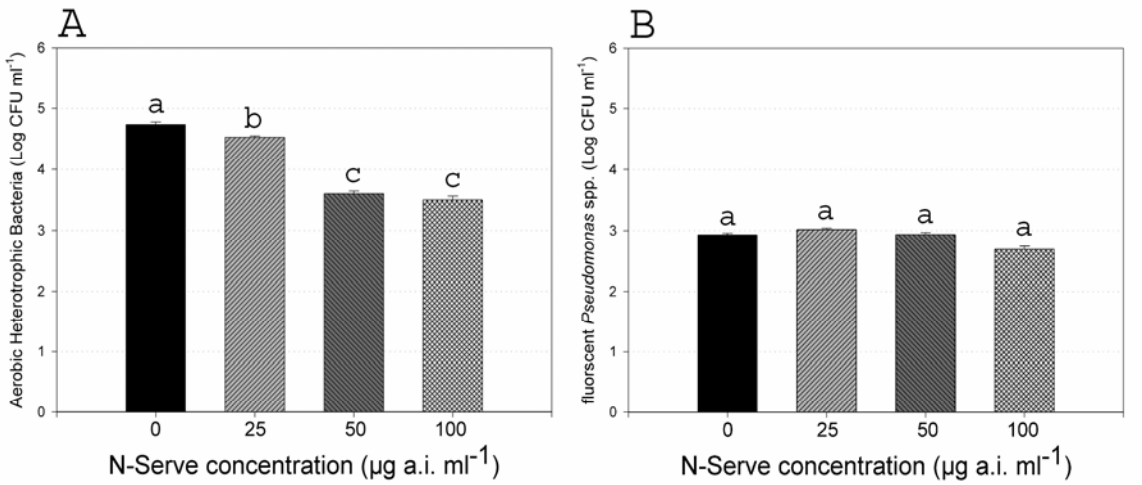

Figure 1.6. Effect of N-Serve® at 25, 50 and 100 µg a.i. ml^{-1} on population densities of indigenous aerobic heterotrophic bacteria (A) and fluorescent pseudomonads (B) after 30 minutes. Nutrient solution samples from greenhouse gerbera experiment were treated *in vitro*. AHB populations were determined by plating a dilution series of each treatment onto Tryptic soy agar and fluorescent pseudomonads by plating onto King's B agar followed by incubation at 25 °C for two days. Values are the means of three replicates. Bars with different letters are significantly different at P = 0.01 according to the All Pairwise Multiple Comparison Procedures (Holm-Sidak method). Error bars represent the standard error of the mean (n = 3) where variation was great enough to be presented.

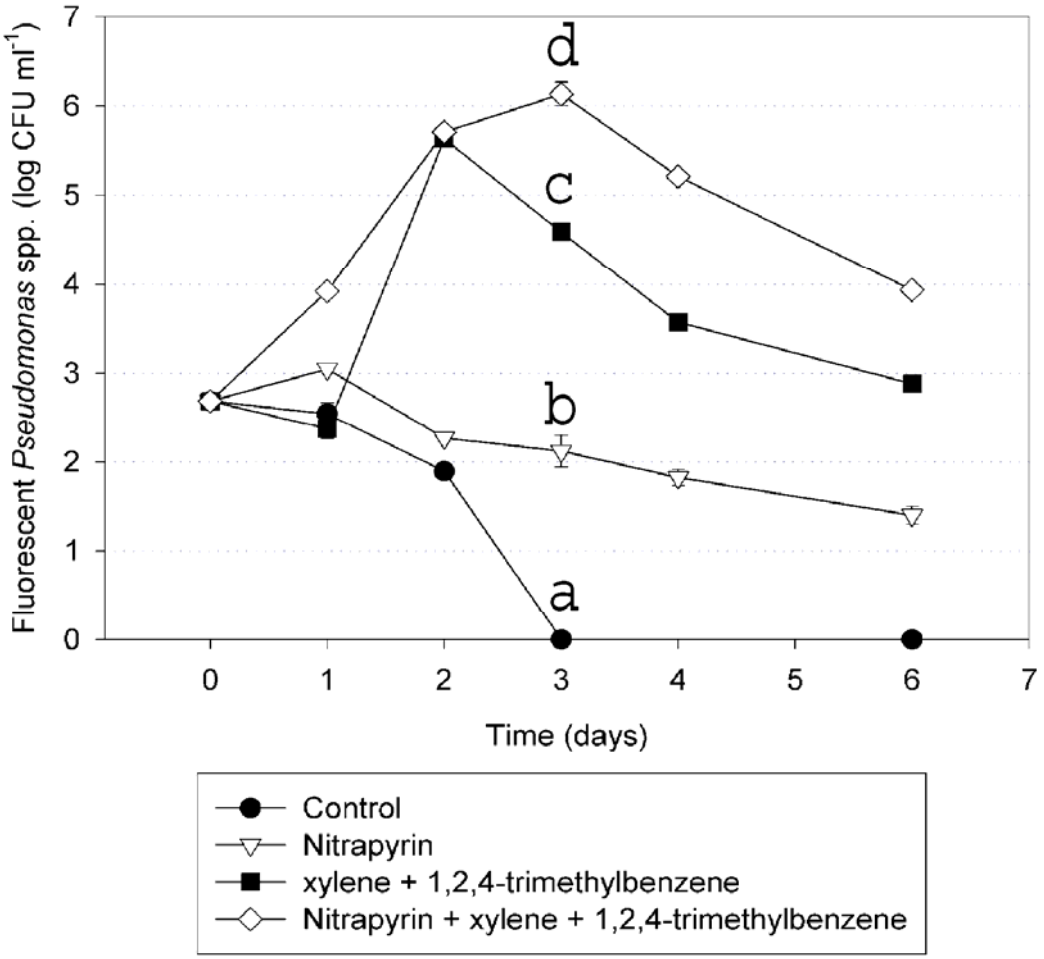

Figure 1.7. Mean population densities of indigenous fluorescent pseudomonads in nutrient solution collected from hydroponic pepper recirculating system. Nutrient solution samples were treated *in vitro* and compared with controls. Treatments consisted of control nutrient solutions receiving no chemical, and/or amended with nitrapyrin (12.5 µg a.i. ml^{-1}) and/or xylene and 1,2,4-trimethylbenzene (20 + 20 µg ml^{-1}) at 0 time. At 3 days time, points with different letters are significantly different at P = 0.01 according to the All Pairwise Multiple Comparison Procedures (Holm-Sidak method). Error bars represent the standard error of the mean (n = 3) where variation was great enough to be presented.

Table 1.2. Mean populations of fluorescent pseudomonads Gerr in sterile distilled water (SDW) and sterile gerbera hydroponic nutrient solution (SNS) amended or not amended with N-Serve® at 25 µg a.i. ml^{-1}.

Treatments [a]	Bacterial concentration (Log CFU ml^{-1}) [b]					
	Incubation period (hours)					
	0	0.5	24	48	72	96
SDW + *P. putida* Gerr [c]	4.40	4.32	3.98	2.79	2.08	--
SDW + *P. putida* Gerr + N-Serve®	4.40	3.19	0	0	0	--
SNS + *P. putida* Gerr	4.40	--	--	1.30	1.30	1.30
SNS + *P. putida* Gerr + N-Serve ®	4.40	--	--	5.06	5.98	4.90

[a] N-Serve® (25 µg a.i. ml^{-1}) was applied to 500 ml of sterile distilled water (SDW) or sterile nutrient solution (SNS).
[b] Mean of three replicates samples.
[c] *Pseudomonas putida* Gerr strain was added at ±4.40 log CFU ml^{-1}.

Table 1.3. Bacterial population densities of pure cultures of strains in nutrient solution amended with N-Serve®.

Treatments [a]	Bacterial concentration (Log CFU ml^{-1}) [b]	
	Incubation period (hours)	
	0 hour	24 hour
N-Serve®	ND [d]	ND
Aeromonas hydrophila isolate 32 [c]	3.12 a [e]	3.09 a
Aeromonas caviae isolate 34	3.23 a	3.18 a
Pseudomonas spp. isolate 36	3.17 a	3.09 a
N-Serve® + *Aeromonas hydrophila* isolate 32	3.10 a	5.36 c
N-Serve® + *Aeromonas caviae* isolate 34	3.22 a	5.01 b
N-Serve® + *Pseudomonas* spp. isolate 36	3.19 a	5.32 c

[a] N-Serve® (12.5 µg a.i. ml^{-1}) was applied to 200 ml of sterile hydroponic nutrient solution.
[b] Mean of three replicates samples.
[c] Strains were previously isolated from hydroponic nutrient solution from a pepper greenhouse experiment.
[d] No bacteria were detected in sterile hydroponic nutrient solution amended with N-Serve®.
[e] Value followed by the same letter are not significantly different at P = 0.05

Table 1.4. Population densities of a bacterial mixture in nutrient solution amended with N-Serve®.

Treatments [b]	Total population (Log CFU ml^{-1}) [a]		Fluorescent *Pseudomonas* spp. population (Log CFU ml^{-1})	
	Incubation period (hours)			
	0 hour	24 hour	0 hour	24 hour
N-Serve®	ND [c]	ND	ND	ND
Bacterial Mixture [d]	4.43 b	4.40 b	3.74 a	3.70 a
N-Serve®+ Bacterial Mixture	4.47 b[e]	5.93 c	3.76 a	5.88 c

[a] Mean of three replicates samples.
[b] N-Serve® (12 µg a.i. ml^{-1}) was applied to 200 ml of sterile hydroponic nutrient solution.
[c] No bacteria were detected in sterile hydroponic nutrient solution amended with N-Serve®.
[d] Bacterial mixture was made of *Aeromonas hydrophila* isolate 32, *Aeromonas caviae* isolate 34 and fluorescent *Pseudomonas* spp. isolate 36. Each strain was added at ±1.3 log CFU ml^{-1} concentration.
[e] Value followed by the same letter are not significantly different at P = 0.05.

CHAPTER 2

CHEMO-BIOLOGICAL SUPPRESSION OF ROOT-INFECTING ZOOSPORIC PATHOGENS IN RECIRCULATING HYDROPONIC SYSTEMS

Deborah Pagliaccia, Donald Ferrin, Michael Eugene Stanghellini

(Submitted to Microbial Ecology)

ABSTRACT

Zoosporic root-infecting pathogens are the most destructive organisms in enclosed environment agricultural systems which recycle irrigation water. Numerous strategies have been proposed for reducing or eliminating these pathogens from recycled irrigation water. These strategies include the following: filtration, sedimentation, chlorination, ozonation, heat, ultraviolet light, application of antimicrobial chemicals, suppressive potting substrates, and biological control agents. The latter strategy has been the focus of numerous investigations but consistency in performance in disease abatement following their application has not been realized. This has been attributed, in part, to the inability of these potential biocontrol bacteria to maintain a critical threshold population necessary for sustained biocontrol activity. That threshold population has been estimated at not less than 10^6 CFU g^{-1} of soil or root. Recently, we demonstrated that amending recycled irrigation water with specific carbon substrates (i.e., N-Serve® or Truban®) resulted in the selective enhancement of the indigenous fluorescent pseudomonad populations to levels at or above the presumed threshold levels necessary for disease control. In our current study, we verified the ubiquity of that response in different cultural systems involving two different host plants (i.e., cucumber and pepper) and, most significantly, we document, for the first time, significant and sustained disease abatement.

Additionally, we demonstrated that nitrapyrin, the active ingredients in N-Serve® and Truban®,exerted direct antifungal activity whereas the inert ingredients had an indirect role in disease suppression. Specifically, the inert ingredients were responsible for the increase in the fluorescent pseudomonad population. Amending the recirculating nutrient solution with a representative fluorescent pseudomonad isolate verified and substantiated their role in disease control.

Cumulatively, our results support the hypothesis that it is possible to modify the environment to make it more conducive to the multiplication and survival of indigenous biocontrol bacteria. The resident microflora in many, if not most, habitats already contain prospective biological control members who, if enhanced, could function as antagonists to other microorganisms in the same habitat.

INTRODUCTION

A major concern in closed hydroponic production systems is the spread of root-infecting zoosporic pathogens in the recycled nutrient solution. These pathogens occur primarily in the following genera: *Pythium*, *Phytophthora*, *Plasmopara*, and *Olpidium*. These plant pathogenic microorganisms produce a motile stage known as zoospores, which are favored by the aquatic environment of the recirculating hydroponic system [31].

Inhibition of these pathogens has been attempted using numerous strategies including filtration, sedimentation, chlorination, ozonation, heat, and ultraviolet light [9]. These methods are known to reduce the pathogenic microflora but might also suppress some other beneficial resident microflora [5]. Nowadays, a change in the way of thinking about disinfections and disinfestations has emerged. Elimination of pathogens by sterilizing the nutrient solution has been questioned. It is perhaps not advantageous to kill all life in the nutrient solution; there is a certain microflora present which may play a role in suppressing disease [15, 23, 24, 35]. Since beneficial micro-organisms also occur in soilless systems, it may be more effective to find a way to increase the beneficial microbes, most likely by manipulating the indigenous microflora in the nutrient solution and rhizosphere [22, 36]. Nutritional amendments such as chitin, amino acids, methionine, olive oil, and salicylate, have been used to selectively enhance colonization and population size of bacterial inoculants [4, 10, 12, 21, 32, 41, 42, 43].

The use of microbial inoculants to control disease has emerged as one of the important methods in the management of soilborne plant pathogens, but their use is still limited in agricultural practice because of the inconsistency of their performance, i.e. insufficient colonization of the infection site or maintenance of a critical threshold population necessary for sustained biocontrol activity. Several fluorescent *Pseudomonas* spp. have historically been associated, via diverse mechanisms, with suppression of root diseases caused by numerous fungal and fungal-like pathogens. These mechanisms include nutrient competition, production of antibiotics, siderophores or biosurfactants, as well as induction of systemic resistance (ISR) [1, 2, 16, 38, 39, 40].

While biocontrol agents have potential for disease suppression, they should not be seen as a stand-alone solution (i.e. single management method may not provide adequate control of a plant disease) and could preferably be used in conjunction with other control strategies [11, 27, 34, 44] in an innovative integrated disease management system. The

combination of different strategies constitutes the greatest challenge as it has the potential to suppress the development of disease, to help maintain pesticides and other chemicals at reasonable levels, to reduce the risks to human health and the environment, and most importantly to encourage natural disease control mechanisms.

Recently, the selective enhancement of indigenous fluorescent pseudomonads after amending the recirculating nutrient solution of a hydroponic system with 25 µg a.i. ml^{-1} of the antimicrobial, nitrification inhibitor N-Serve®24 [24] (Nitrapyrin a.i., Dow AgroSciences; described below) was demonstrated [Pagliaccia *et al*., (2004) Abstract Phytopathology 94]. The enhanced bacterium was identified as a *Pseudomonas putida* strain. Additionally, terminal restriction fragment length polymorphism (T-RFLP) analysis of 16S rDNA suggested that N-Serve®, which selectively increased the *Pseudomonas putida* population, also produced a shift in the bacterial population by reducing bacterial diversity [Pagliaccia *et al*., (2004) Abstract Phytopathology 94; 19].

The overall objectives of the present study were 1) to verify the ubiquity of the selective enhancement of the indigenous fluorescent pseudomonad population following the addition of chemical amendments (N-Serve® and Truban®, both nitrification inhibitors) to the recirculating nutrient solution of different hydroponic cultural systems, 2) to assess the efficacy of these amendments for management of root disease of pepper and cucumber caused by *Phytophthora capsici* and *Pythium aphanidermatum*, respectively, 3) to examine nitrapyrin (N-Serve® active ingredient), xylene and 1,2,4-trimethylbenzene (N-Serve® and Truban® inert ingredients), alone and in combination, for their i*n vitro* effect on mycelial growth of *P. capsici* and *P. aphanidermatum*, and 4) to assess the efficacy of nitrapyrin versus *Pseudomonas putida*, alone and in combination, for management of root disease of cucumber and pepper caused by *P. aphanidermatum* and *P. capsici*.

MATERIALS AND METHODS

Hosts and pathogens. Two host plants, pepper (*Capsicum annuum* L. cv. Joe Parker) and cucumber (*Cucumis sativus* L. cv. Corona) were utilized to assess the responses of different plant species to changes in indigenous bacterial populations following chemical amendments of the nutrient solution. Plant survival rates were assessed after inoculation with *Phytophthora capsici* L. and *Pythium aphanidermatum* (Edson) Fitzp., respectively, used as root pathogens. Stock cultures of *P. capsici* and *P. aphanidermatum* were stored in sterile distilled water, and working cultures were grown on 20% and 10% clarified V8 juice agar (V8C), respectively.

Hydroponic cultural systems. Experiments were conducted in temperature-controlled greenhouses (20-32°C) using three different recirculating hydroponic systems: ebb and flow (Jetflo™, American Agritech, Tempe, AZ) (Fig. 2.1B), drip irrigation (Jetstream™, American Agritech) (Fig. 2.1A), and Phototron 2 floating systems (Pyraponic Industries, Los Angeles, CA, 90061) (Fig. 2.1C). Ebb and flow system and drip irrigation systems, consisting of recirculating hydroponic units with an upper container for plant growth, and a lower reservoir where the nutrient solution is kept and recycled. In the drip irrigation system, plants are top irrigated with emitters; in the ebb and flow system, plants are watered from the bottom (benches are flooded with nutrient solution that is taken up by the potting medium via capillary action). Plants were irrigated for 30 minutes, 3 to 5 times during a 24-h period (3h interval - 8 a.m. and 8 p.m.) depending of the season. The floating system consisted of units with styrofoam boards with 12 holes each to hold plants, which were floated on top of 12 L of aerated nutrient solution in the reservoir of a Phototron 2 growth chamber. Unless otherwise specified, all cultural practices were the same as those employed in previous investigations [17, 18, 33].

In pepper experiments, three-week-old seedlings, grown on Oasis Horticubes (Smithers-Oasis, Kent, OH) in a temperature-controlled incubator at 25°C, were transplanted into either a commercial organic (peat-based) potting mix (Supersoil®, Rod McLellan Company, San Mateo, CA) in 11-cm×11-cm×12-cm plastic pots or directly placed in holes (25-mm diameter) cut into the styrofoam boards of the floating system. In cucumber experiments, three-day-old seedlings grown on Grodan Rockwool (2.5 x 5 cm)

in a temperature-controlled incubator at 30°C were transplanted into rock wool slab (10 cm^3) (Saint-Gobain Cultilène B.V., Tilburg, The Netherland).

Six (in pepper experiments) or eight (in cucumber experiments) potted plants (at 4-leaf stage) were placed in each unit of the drip irrigation system, with 12 cm between plants; 30 plants were placed in the ebb and flow systems, with 12 cm between plant, and 11 to 12 plants in the floating system, with 6 cm between plants.

Treatments. Six experiments with seven different treatments were carried out. The number of treatments and treatment replications per experiment are summarized in Table 2.1. Two chemicals were used as amendments to the recirculating nutrient solutions: N-Serve® 24 (Nitrapyrin a.i., Dow AgroSciences, Indianapolis), a commercial nitrification inhibitor, and Truban®25E (Etridiazole a.i., The Scotts Company) a contact fungicide for the control of damping-off, root and stem rot disease in ornamental and nursery crops. Etridiazole, the active ingredient of Truban®25E, acts also as nitrification inhibitor and it is marketed under the trade names Dwell® or Terrazole® (Uniroyal Chemical Company). The commercial product N-Serve®24 contains 22.2% nitrapyrin, 2.25% related chlorinated pyridines, and 75.55% inert ingredients (23% 1,2,4-trimethylbenzene and 2% xylene-mixed isomers are the primary inert ingredients). Truban® 25EC contains 25% etridiazole and 75% inert ingredients (15% xylene-mixed isomers is the primary inert ingredients).

In the experiments in the ebb and flow and drip irrigation systems, N-Serve® and Truban® were added to the nutrient solution 1h before the start of the irrigation. In the experiment with floating systems, the nutrient solution was treated with the chemicals while the plants were kept outside the systems. After 1h the plants were placed back in the nutrient solution. The nutrient solution was treated with each chemical at 10-day intervals. Pathogens were introduced into the system 9 days after the first chemical application, or between the second and the third chemical application depending on the experiment. After completion of an experiment, the entire hydroponic system in the greenhouse was surface sterilized with sodium hypochlorite (0.6%).

Pathogen inoculation technique. Pathogen infestation of an individual hydroponic unit was achieved by hypocotyl inoculation of a single plant from each system. This method of infestation permitted the evaluation of pathogen spread within a unit following colonization and natural reproduction of the pathogen on the roots of the inoculated plants. Specifically, in drip irrigation and ebb and flow systems, a 10-mm-diameter disk, cut from

the advancing margin of a 5-day-old V8 agar culture (for *P. capsici*) and a 3-day-old V8 agar culture (for *P. aphanidermatum*) was placed in contact with the lower hypocotyl (immediately below the substrate surface) of one pepper or cucumber plant, respectively. In floating systems, inoculation was conducted when plants were 50-60 days old as follows: one plant was removed from each treatment and the roots of that plant were placed in a glass beaker containing 100 ml of water infested with 10^3 zoospores ml^{-1}. After a 30-minute incubation period, the seedling was removed from the zoospore suspension and re-inserted in the phototron to serve as a source of inoculum for the rest of the plants. *P. capsici* zoospores were obtained by placing four V8 agar disks (each 10-mm-diameter) from the edge of a 7-day-old cultures into Petri dishes (9-cm-diameter) containing 15 ml of sterile distilled water for 1-2 h to allow release of zoospores from sporangia. The zoospore suspension was obtained by filtering the water-cultures through a 25-µm sieve. Numbers of zoospores were estimated with a hemacytometer (Hausser Scientific; Horsham, PA).

Monitoring fluorescent pseudomonad populations in the nutrient solution and in the rhizoplane. Twenty milliliter samples of the nutrient solution were collected at different intervals (primarly taken at 0, 24, 48 and 72 hours after chemical amendment) from the reservoir of each treatment. After serial 10-fold dilutions, aliquots were plated in triplicate, using a spiral plater (Autoplate 4000 – Spiral Biotech, Inc) onto 10-cm-diameter Petri dishes containing King's B medium (KB) for enumeration of fluorescent pseudomonads. KB agar was supplemented with 50 µg ml^{-1} cycloheximide (Calbiochen) and 75 µg ml^{-1} penicillin (Calbiochen). Colonies were counted under UV light after 24 h incubation at 28° C.

The effects of N-Serve® and Truban® amendments to the nutrient solution on the rhizoplane population of fluorescent pseudomonads (i.e. root colonization) were investigated in the pepper greenhouse experiments with both drip and ebb and flow irrigation and compared to rhizoplane fluorescent pseudomonad populations in the un-amended treatments. Ten days after the last application of the amendments, two plants were randomly chosen from each treatment. The roots were excised, blotted to remove excess water and weighed. The roots, 0.5 g, were then placed in 10 ml sterile distilled water and agitated in a shaker (200 rpm) for 1 h at 28°C. After serial 10-fold dilutions, aliquots of the suspension were plated, in triplicate, onto King's B medium (KB) as described

above for enumeration of the fluorescent pseudomonads. The experiment was repeated twice.

Survival of plants in the absence or presence of N-Serve® or Truban®. Disease incidence (i.e., pepper and cucumber plant mortality after pathogen introduction) was monitored daily throughout the duration of each experiment. The data set of this study contained, however, many censored observations (i.e., plants that had not died by the end of the assessment period); therefore time to death, the dependent variable, was interpreted as a "length of survival", and survival analysis techniques were used to described and model the data. The application of survival analysis has been limited in plant pathology. In the last few years however, this model have been successfully employed by other researchers [6, 29, 30].

At the end of each experiment, five root segments, each 1-2 cm long, were excised from three plants from each treatment and plated onto PARPH medium for *P. capsici* and water agar for *P. aphanidermatum*. After 72 h incubation at 28° C, hyphae emerging from the roots were transferred to clarified V8 agar medium, and the cultures were identified.

Correlation between the fluorescent pseudomonad population, chemical treatments and disease control (i.e., plant survival time). To examine the relationship between the fluorescent pseudomonad population, chemical amendments (treatments) and disease control (i.e., plant survival time), Spearman correlation coefficients (r_s) were calculated in all experiments (i.e., fluorescent pseudomonads versus treatments, fluorescent pseudomonads versus disease control, and treatments versus disease control).

Growth chamber and greenhouse studies on plant survival after nutrient solution amendments with nitrapyrin or *Pseudomonas putida.* A small scale growth chamber study was performed to determine if disease control of cucumber plants could be mediated by *Pseudomonas putida* pep4 at $5x10^6$ CFU ml^{-1} or nitrapyrin at 12.5 µg ml^{-1} of nutrient solution respectively, when used as a treatment alone and combine as a mixture. Isolate *Pseudomonas putida* pep4 was chosen for the bacterial treatment. This isolate was representative of the fluorescent pseudomonad population that was selectively enhanced by N-Serve® amendment of the nutrient solution.

Experiments were conducted using cucumber (*Cucumis sativus* L. cv. Corona) as the susceptible host, and *P. aphanidermatum* as the pathogen. Cucumber seeds were sterilized in a NaClO solution (1%, v/v) for 5 minutes, washed with ethanol, then with sterile distilled water and placed to germinate on Grodan Rockwool blocks (3.8x3.8x3.8 cm) (Saint-Gobain Cultilène B.V., Tilburg, The Netherland) in a temperature-controlled incubator at 28°C. Three-day-old seedlings were then placed in magenta GA-7 vessels (7.6×7.6×10.2 cm, Magenta Corp. Chicago, Ill) and grown in a temperature-controlled incubator at 30°C with a 16-h photoperiod. There were 3 replicate vessels per treatment with four seedlings each for a total of 12 seedlings per treatment. Treatments consisted of fresh nutrient solution treated with *Pseudomonas putida* pep4 at $5x10^6$ CFU ml^{-1} or nitrapyrin at 12.5 µg ml^{-1} or a mixture of the two. Inocula of *Pseudomonas putida* pep4 were prepared by growing the isolate first in LB broth (EMD Chemicals Inc.) at 28°C in a shaker (120 r.p.m.) for 24 h, then in KB agar for 48 h. Finally two loops were transferred into 10 ml of 0.01 M $MgSO_4$ solution. These cells were washed twice in $MgSO_4$ and CFU ml^{-1} assessed at 600 nm using Genesys 10vs Spectrophotometer (Spectronic Unicam). These suspensions (10^8 CFU ml^{-1}) were then diluted to desired population densities for the study. Controls consisted of a non-amended nutrient solution. Seedlings received three applications of each treatment (100 ml per vessel at day 4, 11 and 18). Inoculation with *P. aphanidermatum* zoospores occurred the day before the 3rd application (day 17th). Each vessel was infested with 600 zoospores of *P. aphanidermatum. P. aphanidermatum* zoospore inoculum was obtained with the method described above for *P. capsici*, but using 2-day-old cultures and 24 h incubation period at room temperature. Plants mortality was recorded six days after inoculation. The experiment was repeated 2 times.

Additionally, a greenhouse experiment was performed to verify the results from the growth chamber study regarding disease suppression mediated by the active ingredient alone (nitrapyrin) versus the bacterium alone (*Pseudomonas putida*). Experiments were conducted in a temperature-controlled greenhouse (20-32°C) using a recirculating hydroponic system with drip irrigation (Jetstream™, American Agritech) as described previously. Experiments were conducted using pepper (*Capsicum annuum* L. cv. Joe Parker) as the susceptible host, and *Phytophthora capsici* Leonian as the root-infecting pathogen.

There were 2 experiments and each experiment consisted of 4 treatments with eight plants per treatment-unit: nitrapyrin (90%, N-ServeTG®) at 12.5 µg a.i. ml^{-1}; *Pseudomonas putida* pep4 applied at $5x10^6$ CFU ml^{-1}; and a mixture of nitrapyrin and *Pseudomonas putida* pep4. Inocula of *Pseudomonas putida* pep4 were prepared as described above. Nutrient solution with no chemical or no bacteria amendments served as the controls. Treatments were first applied to the nutrient solution 3 days after the start of the experiment and reapplied four times at 7 day intervals. Inoculation with *P. capsici* was conducted one day before the 4^{th} chemical treatment, when plants were 50 days old, as previously described. Disease incidence (i.e., - pepper plant mortality after pathogen introduction) was monitored daily throughout the duration of each experiment and survival analysis techniques were used as described previously to model the data.

Evaluation of antifungal activity of N-Serve® components and Truban®. The active ingredient of N-Serve®, nitrapyrin (90% a.i., N-ServeTG®; Dow AgroSciences, Indianapolis), and the inert ingredients of N-Serve®, xylene (99.7%, Mallinkrodt Baker, Inc.) and 1,2,4-trimethylbenzene (98%, Sigma-Aldrich), either alone or in combination, were screened for antifungal activity against *Phytophthora capsici* and *Pythium aphanidermatum* in an amended agar method (Table 2.3). The commercial product Truban® (etridiazole a.i., The Scotts Company) was also tested.

Five 500-ml Erlenmeyer flasks, each containing 250 ml of 10% clarified V8 juice agar (V8C), were autoclaved and cooled to approximately 55°C. The V8 agar was then amended with chemicals. There were five different treatments: nitrapyrin at 12.5 µg ml^{-1}; 1,2,4-trimethylbenzene at 20 µg ml^{-1} plus xylene at 20 µg ml^{-1}; a mix of nitrapyrin, 1,2,4-trimethylbenzene and xylene, Truban® at 12.5 µg a.i. ml^{-1}, and a control receiving sterile distilled water as the amendment. Media were dispensed in 12-ml aliquots into disposable Petri dishes (90 × 15 mm). Once the media solidified, a 5-mm agar plug, from the margin of a 4-day-old and 2-day-old V8A colony of *Phytophthora capsici* or *Pythium aphanidermatum*, respectively, was placed in the margin of each dish. Four replicate dishes were used for each treatment and unamended control. Cultures were incubated in the dark at 25°C. Diameters of mycelium growth of individual colonies were measured after unamended control colonies had reached 100% growth on the Petri dishes: three days for *Pythium aphanidermatum* and seven days for *Phytophthora capsici*. Percent growth was

calculated dividing the diameter of each colony by the mean diameter of the unamended control colonies. These assays were conducted twice.

Statistical analysis. Plant death from root rot (caused by *P. capsici* or *P. aphanidermatum*) was the primary end point and, for every treatment, was assessed by plotting survival curves according to the Kaplan-Meier method [30]. The data were analyzed with a closeout (study censor) date opted to be 10 days after the last plant had died in the *P. capsici* or *P. aphanidermatum* treatments. Data were considered censored when plant had not died by the end of the closeout date. In each of the six experiments, the differences between the survival curves were tested for statistical significance with the log-rank test followed by a multiple comparison procedure (Holm-Sidak method) to isolate the treatments that differ from the others. Homogeneity test of data from two repeated experiments was evaluated by Cox Proportional Hazard model using a Breslow test at P=0.05. If data, from separate trials, did not differ significantly, they were pooled and analyzed as described above.

The spearman Rank Order Correlation test was used to measure the strength of association between fluorescent pseudomonad populations, treatments and disease control (i.e., plant survival time). This statistical method was chosen because the data did not have a normal distribution and there were two dependent variables: the fluorescent pseudomonad populations and plant survival time. Fluorescent pseudomonad populations and plant survival time data were tested for normality using the Kolmogorov-Smirnov test at P=0.05. For analysis purposes, in each experiment, fluorescent pseudomonad population data were obtained by estimating bacterial population numbers 72 hours after each chemical application. The latter time interval corresponded to the highest fluorescent pseudomonads population (i.e. – lowest data points were not included in the analysis because they have no relevance for biological control).

Data from the evaluation of antifungal properties of N-Serve® components were found to be non-normal; therefore data from two repeated experiments were analyzed by the non-parametric test Kruskal-Wallis one way analysis of variance, followed by the All Pairwise Multiple Comparison Procedures (Student-Newman-Keuls Method) at P=0.01.

Data from the fluorescent pseudomonad root colonization study in the greenhouse and the small scale growth chamber study on plant survival after nutrient solution amendments with nitrapyrin and/or *Pseudomonas putida* were analyzed by the Kruskal-

Wallis One Way Analysis of Variance on rank, followed by the All Pairwise Multiple Comparison Procedures (Student-Newman-Keuls Method) (P=0.05). Fluorescent pseudomonad population data were $\log_{10}$ transformed before statistical analysis.

All data were analyzed using SigmaStat 3.0 statistical software package (SPSS Science, Chicago, IL). All experiments were repeated at least once.

RESULTS

Monitoring fluorescent pseudomonad populations in the nutrient solution and in the rhizoplane. The population dynamics of the indigenous fluorescent pseudomonads in recirculating nutrient solutions amendment with N-Serve® were monitored in different hydroponic systems used in the cultivation of pepper and cucumber plants. In all experiments, significant population increases were consistently recorded in N-Serve®-amended compared to non-amended nutrient solutions (Fig. 2.2B, 2.3B, 2.4B and 2.5B). The bacteria population increases, which were two or more orders of magnitude higher than their respective controls, occurred within 48 to 72 h after each consecutive addition of N-Serve®. Similar results were obtained with Truban® (Fig. 2.2B and 2.5B).

Fig. 2.6A and 2.6B shows the fluorescent pseudomonads population present in the rhizoplane in the two hydroponic systems. N-Serve® and Truban® treatments affected the fluorescent pseudomonads population on the root of pepper plants in both drip irrigated and ebb and flow systems. Specifically, fluorescent pseudomonad populations (CFU g^{-1} of roots) in the drip irrigated system were significantly higher (P= 0.002) in both N-Serve® and Truban® treated nutrient solution compare to the un-amended solution. Significantly higher (P=0.001) fluorescent pseudomonad populations on the roots were also detected in the ebb and flow system of both N-Serve® and Truban® treated nutrient solution compare to the un-amended solution. Additionally, we noted that the fluorescent pseudomonads recovered from amended nutrient solutions, or from roots from amended systems, exhibited greater fluorescence than the fluorescent pseudomonads from un-amended systems when grown on KB medium (Fig. 2.6).

Survival of plants in the absence or presence of N-Serve® or Truban®. All hypocotyl-inoculated pepper and cucumber plants (which served as source of inoculum for spread to

other plants in the recirculating system), with a single exception (Fig. 2.5A), in all experiments and all treatments wilted and died within 10 days after inoculation (Fig. 2.2A, 2.3A, 2.4A and 2.5A). In the absence of N-Serve® or Truban®, all non-inoculated pepper plants died within the next 53 days in an ebb and flow hydroponic recirculating unit (Fig. 2.2A), within 28 days in the drip irrigated systems (Fig. 2.3A), and within 10 days in the floating systems (Fig. 2.4A). In the cucumber experiments, in the absence of N-Serve® or Truban® all the non-inoculated plants died within 24 days (Fig. 2.5A). In contrast, amending the nutrient solution with N-Serve® or Truban® resulted in a significant increase in the survival of both pepper and cucumber plant in all the different hydroponic systems (Fig. 2.2A, 2.3A, 2.4A and 2.5A). No plants died in the non-inoculated treatments.

Although similar trends in plant survival were recorded following amendment of the nutrient solution with either N-Serve® or Truban®, the rapidity of suppression varied between the different types of hydroponic systems used. Fig. 2.2A, 2.3A, 2.4A and 2.5A show the Kaplan-Meier estimates of the probability of death from root rot in the different experiments with and without chemical treatments and with and without selective enhancement of fluorescent pseudomonad population. Among pepper plants treated with N-Serve® or Truban® in the ebb and flow system (Fig. 2.2A), the risk of root rot caused by *P. capsici* decreased within few days after inoculation to about 2 % (the only dead plant was the inoculated one) with no progress in plant death over the 53 days experiment. Among pepper plants grown in the drip irrigated system (Fig. 2.3A), plants mortality due to root rot occurred gradually after pathogen inoculation and reached 100% within four weeks in the control. Disease suppression was also related to the frequency of application of the chemical amendments. Plant survival with a single application of N-Serve® (Fig. 2.3A) was significantly greater compare the control (P=0.02), but produce only a shift in the curve (i.e. plants loss delay in time) with 100% loss in 30 days. However, four applications of N-Serve® showed a significant survival advantage (75%) compared to both the control (P <0.0007) and the one- N-Serve® application treatment (P=0.002). In the floating growing system, all the Kaplan-Meier survival curves are displaced toward shorter survival (Fig. 2.4A) with plant loss occurring early after pathogen inoculation (within the first 2 weeks), with a significant better survival for the N-Serve® amended treatments compare to the control (P <0.001). No significant difference appeared between the two- or three-N-Serve® application survival curves (P=0.1).

Fig. 2.5A shows the Kaplan-Meier estimates of the probability of cucumber plant death from root rot caused by *P. aphanidermatum* in the drip irrigated system with and without chemical treatments. Truban® treatments at 12.5 µg ml^{-1} significantly increased the survival rate (100% on both repeated experiments) compared to the 0% survival of the control treatment 32 days after pathogen inoculation. In contrast, even though N-Serve® at 12.5 µg ml^{-1} induced a significant (P=5.2x10^{-8}) in the 1st experiment and P=3x10^{-8} in the 2nd experiment) longer cucumber survival time compare to the control, plant death due to root rot reached 100% within 27 days.

Correlation between fluorescent pseudomonad population, treatments and disease control (i.e., plant survival time). Spearman correlation coefficients (r_s) are presented in Table 2.2 for three comparisons: fluorescent pseudomonad population versus treatments, fluorescent pseudomonad population versus disease control, and treatments versus disease control. For all the hydroponic systems studied, there were high, statistically significant correlations for all these comparisons (r_s = 76; range, 0.51-0.93), with the exception of two comparisons in the pepper experiments with drip irrigation and one N-Serve® application: fluorescent pseudomonad versus disease control, and treatments versus disease control. The strongest correlations were found for fluorescent treatments versus pseudomonad populations comparisons (r_s = 0.89-0.90) regardless of N-Serve® or Truban® application, which indicates that the variability of population densities in the treated solution with either N-Serve® or Truban® was very low. Good to strong correlations were also found among fluorescent pseudomonad populations versus disease control (range, r_s = 0.58-0.93), and treatments versus disease control (range, r_s = 0.51-0.80).

Growth chamber and greenhouse studies on plant survival after nutrient solution amendments with nitrapyrin or *Pseudomonas putida.* The results from the small scale growth chamber study suggested the possible involvement of both nitrapyrin (the N-Serve® active ingredient), and the selectively-enhanced fluorescent pseudomonad population in disease suppression. The probability of cucumber plants dying from root rot after treatment of the nutrient solution with either nitrapyrin or *Pseudomonas putida* alone was significantly reduced compared to the control (Fig. 2.7). No significant difference (P=0.17) was observed between nitrapyrin and the *Pseudomonas putida* pep4 treatments (Fig. 2.7).

However, amendment of nutrient solution with both nitrapyrin and *Pseudomonas putida* significantly increase plant survival in relation to all other treatments.

The results from the greenhouse experiment suggested and confirmed the possible involvement of the selectively-enhanced fluorescent pseudomonads in disease suppression. Fig. 2.9 shows the Kaplan-Meier estimates of the probability of pepper plants death from root rot caused by *P. capsici* in the drip irrigation system. The probability of pepper plants dying from root rot after 4 treatments of the nutrient solution with *Pseudomonas putida* at $5x10^6$ CFU ml^{-1} was significantly reduced (P=0.002) compared to the control (Fig. 2.9). Because of the high toxicity to the plants (Fig. 2.8) that compromised the pathogen spread between plants, amendment of nutrient solution with nitrapyrin was not considered for the statistical comparison versus the other treatments.

Evaluation of antifungal activity of N-Serve® components and Truban®. The mycelium growth of both *P. capsici* and *P. aphanidermatum* was significantly reduced in the presence of nitrapyrin or nitrapyrin plus xylene plus 1,2,4-trimethylbenzene (Table 2.3). No significant growth inhibition (0%) of *P. aphanidermatum* occurred with the solvents (xylene plus 1,2,4-trimethylbenzene) alone while a slightly significant difference in mycelium growth occurred with the solvents (6%) compare to the control. Specifically, the growth of *P. capsici* and *P. aphanidermatum* were inhibited by 69% and 46%, respectively, at a concentration of 12.5 µg $ml^{-1}$of nitrapyrin compare to the control treatment. Adding nitrapyrin, xylene and 1,2,4-trimethylbenzene together did not significantly increase the mycelium inhibition (79% for *P. capsici* and 53% for *P. aphanidermatum*) compare to nitrapyrin treatment alone (69% and 46%). Truban® at 12.5 µg a.i. ml^{-1} significantly inhibited growth of *P. aphanidermatum* (81%) and *P. capsici* (72%) compare to the control treatments. However after seven days incubation further mycelium growth (100%) had occurred on plates containing Truban® at 12.5 µg a.i.1^{-1}.

DISCUSSION

Our previous studies [19] on the selective enhancement of an indigenous fluorescent pseudomonad population following application of N-Serve®, a nitrogen stabilizer, to recycled nutrient solutions was verified and extended in this investigation. Specifically, the latter phenomenon proved to be consistent in all experiments (Fig. 2.2B, 3B, 4B and 5B) regardless of the hydroponic system employed (ebb and flow, drip irrigation or floating), plant species used as the host (pepper or cucumber), or the substrate utilized (peat, rockwool or no substrate). Similar results were also recorded following the addition of N-Serve® to the nutrient solution in a recirculating system from a commercial ornamental plant (primarily poinsettia and Gerbera) nursery (Albani & Ruggieri, Civitavecchia) in Italy (senior author, unpublished data).

Concomitant with the selective enhancement of the indigenous fluorescent pseudomonad population, and herein reported for the first time, was a decrease in the onset and severity of root rot caused by *Pythium* and *Phytophthora*. Specifically, the application of N-Serve® provided significant control of rot root in experiments in the ebb and flow hydroponic system (Fig. 2.2A), and a significant delay in the disease progress in both the drip and floating hydroponic systems (Fig. 2.3A, 2.4A and 2.5A). Most importantly, there was a strong correlation between treatments with N-Serve®, increases in the fluorescent pseudomonad population, and disease reduction (Table 2.2).

Our laboratory studies suggested that both the active and inert ingredients in N-Serve® were involved in disease suppression. Specifically, nitrapyrin (the active ingredient) was shown to reduce, to a limited but significant level, vegetative growth of both pathogens (Table 2.3). Although nitrapyrin inhibited vegetative growth of the pathogens, the concentration of the commercial product (N-Serve®) used to amend the nutrient solutions in greenhouse experiments did not prevent pathogen colonization of the deliberately inoculated plants which served as a source of inoculum for pathogen spread in recycled nutrient solution. However, nitrapyrin, when used alone to amend the nutrient solution in greenhouse studies, was very phytotoxic which precluded assessment of the precise role of the nitrayprin in disease suppression (Fig. 2.8 and 2.9).

In contrast to the active ingredient in N-Serve®, the inert ingredients (xylene and 1,2,4-trimethylbenzene), which were previously shown to serve as carbon sources for the selective enhancement of the indigenous fluorescent pseudomonad population in the

recycled nutrient solution [19], had no effect on either of the pathogens (Table 2.3) or phytotoxicity to the plants (data not shown). Furthermore, the selective enhancement of the fluorescent pseudomonad population was shown to be significantly correlated with disease suppression. These results suggested that the fluorescent pseudomonad were either directly or indirectly involved in disease control. The addition of high population of *Pseudomonas putida* alone to the nutrient solution resulted in the reduction of the rapidity of plant mortality relative to the inoculated control treatment, verifying their role. The latter results occurred in both small scale growth chamber (Fig. 2.7) and large scale greenhouse trials (Fig. 2.9). Additionally, to achieve sustained disease control it was necessary to maintain optimal population levels of pseudomonads (10^5 – 10^6 CFU/ml) by adding the amendment more than once (Fig. 2.3A and Table 2.2). Cumulatively, these results suggest that the latter populations, in addition to the active ingredient, play a significant role in disease suppression. However, the specific mechanism is not known but is currently under investigation. It has been suggested that fluorescent pseudomonads may act by producing antibiotics or siderophores, by niche exclusion or by inducing host defense responses in plants [1, 2, 16, 38, 39, 40].

While the biological control approach has been successful in the greenhouse, they have mostly failed in the field. The reason for the failure is most likely due to the inability of the introduced agent to achieve optimal concentrations (assumed to be around 10^5 to 10^6 CFU per g of soil or root) [8, 20] levels that are required for their biocontrol activity due to the adverse soil environment and to the competition from other microorganisms. In our study, we have been able to overcome the problem of establishment of an introduced but foreign biological control agent into the target system, by enhancing a portion of the resident microflora in the nutrient solution by the addition of specific chemicals. Moreover, the presence of high populations of fluorescent pseudomonads on roots (Fig. 2.6A and 2.6B) provide evidence of the ability of the enhanced fluorescent pseudomonads in the nutrient solution to colonize roots. Thus, both the high population of the enhanced fluorescent population in amended nutrient solution coupled with their ability to colonize the rhizoplane, (Fig. 2.2B, 2.3B, 2.4B, 2.5B, 2.6A and 2.6B) may have contributed to disease control. Because of their simplicity, hydroponic systems seem to be more ideal than complex soil systems for the establishment and multiplication of biocontrol agents. It seems reasonable to assume that biological control measures can be achieved more readily

in hydroponic systems [28], compared to the field. Moreover, our system seems to have the advantage of being consistent perhaps because pseudomonads, particularly, *P. putida* always multiply in response to the chemical amendment, regardless of the type of the plant and substrates used in the hydroponic systems.

In our previous study [19], nitrapyrin, when used with its solvent, exhibited a synergistic effect as to its ability to increase the fluorescent pseudomonad population. We considered these traits as having great potential in biocontrol application for plant protection and for the development of a new approach for integrated disease management (IDM). Generally in the traditional IDM strategy, the combination of biorational pesticides (also known as least-toxic or biopesticides) is necessary in order to avoid toxicity to the biological control organism introduced [7, 11, 27, 44]. Our findings indicated that N-Serve®, containing active and inert ingredients, could serve (directly or indirectly) as biological and chemical control at the same time, since they exhibited bi-functional properties. Thus, despite its specific limitation (i.e. phytotoxicity), nitrapyrin can serve as a model system leading to the identification of other potential products for this new IDM strategy. The data from the experiments with Truban® appear to support this assumption. Truban® amendment at 12.5 µg ml^{-1} [i.e. 1/10 of the rate recommended for ornamental and nursery plants (78-156 µg ml^{-1})] significantly increased the population size of *Pseudomonas putida* (Fig. 2.2B and 2.5B). Most importantly the combination of the non-phytotoxic active ingredient (etridiazole) with xylene (the inert ingredient), consistently provided disease suppression of root rot caused by *P. capsici* in pepper experiments with ebb and flow systems (Fig. 2.2A) and a greater reduction of root rot (caused by *P. aphanidermatum*) compared to N-Serve® in cucumber experiments with drip irrigation systems (Fig. 2.5A). Truban®, like N-Serve®, did not completely inhibit vegetative growth of *P. capsici* and *P. aphanidermatum* (i.e., mycelium growth was only temporarily stopped) (Table 2.3) and did not prevent pathogen colonization of the deliberately inoculated plants (with a single exception) which served as a source of inoculum for pathogen spread in recycled nutrient solution. Fungistatic activity of etridiazole, Truban® active ingredient, has been previously reported [26]. Thus, also with Truban® the capacity of rapid bacterial growth in the nutrient solution (Fig. 2.2B and 2.5B) and the higher colonization of the root system (Fig. 2.6A and 2.6B) may also have contributed to disease control.

The mechanism through which the increase in population size of fluorescent pseudomonads resulted in an increase in the level of disease control is uncertain. As discussed above, siderophore production in the rhizosphere and soil by fluorescent pseudomonads can result in antagonism towards other microorganisms [1, 14, 37]. Our observation that N-Serve® or Truban® amendments resulted in increases in fluorescent pseudomonad populations in the nutrient solution (accounting for 80% of total heterotrophic bacterial population, [19]), as well as on the rhizoplane, suggest a need for studies of biocontrol strategies where competition for iron is being utilized to limit the growth and development of pathogenic microorganisms. The stronger visual fluorescence produced on iron depleted substrate (i.e. siderophores-pyoverdines production) by the pseudomonads population selectively enhanced by N-Serve® and Truban® compare to the pseudomonads population in the un-amended solution suggest this possibility. Siderophores could be very effective for disease control in hydroponic systems, where the iron availability to the plant can be controlled and maintained at low concentration by the synthetic chelator added to the nutrient solution [Bakker PAHM, 1998. Abstract 3.8.4S]. The possibility of disease suppression due to siderophore production opens the door to further investigations on both the natural siderophores, produced by the indigenous fluorescent pseudomonad selectively increased in our system, as well as the synthetic siderophore-type chelator such us nitrapyrin-like molecules (for example pyridine-2,6-dithiocarboxylic acid (PDTC) [3]. The combination of the selective enhancement of indigenous fluorescent pseudomonad and the choice of synthetic chelates with a high affinity for iron, which would not be available to the pathogen, could improve disease suppression mediated by siderophores. Leeman *et al*. [13] observed that lowering iron availability for *P. fluorescens* WCS474 and WCS417 increased disease suppression as a result of the ISR triggered by the siderophore produced from these strains. Therefore, if the synthetic chelate chosen to be used in the hydroponic system would also be unavailable to the selectively enhanced indigenous fluorescent pseudomonad, perhaps the fluorescent pseudomonads themselves would be forced to produce even more siderophores.

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Table 2.1. Summary of treatments and replications in each experiment to assess the efficacy of two nitrogen stabilizers in the management of *Phytophthora capsici* and *Pythium aphanidermatum* in hydroponic systems.

Plant **(Hydroponic System)**	**Substrate**	**Treatment**	**# Replication** **(Plants per replication)**	**# of chemical application** [b]
Pepper [a]	Peat	Control	1 (30)	0
(Ebb and flow)		*P. capsici*\	1 (30)	0
		N-Serve 12.5 µg a.i. ml^{-1} + *P. capsici*	1 (30)	4
		Truban 12.5 µg a.i. ml^{-1} + *P. capsici*	1 (30)	4
Pepper	Peat	Control	1 (6)	0
(Drip irrigation)		*P. capsici*	1 (6)	0
		N-Serve 12.5 µg a.i. ml^{-1} + *P. capsici*	1 (6)	1
Pepper	Peat	Control	1 (6)	0
(Drip irrigation)		*P. capsici*	1 (6)	0
		N-Serve 12.5 µg a.i. ml^{-1} + *P. capsici*	1 (6)	4
Pepper	No substrate	Control	1 (12)	0
(Floating)		*P. capsici*	1 (12)	0
		N-Serve 12.5 µg a.i. ml^{-1} + *P. capsici*	1 (12)	2
Pepper	No substrate	Control	1 (11)	0
(Floating)		*P. capsici*	1 (11)	0
		N-Serve 12.5 µg a.i. ml^{-1} + *P. capsici*	1 (11)	3
Cucumber	Rockwool	Control	2 (8)	0
(Drip irrigation)		*P. aphanidermatum*	2 (8)	0
		N-Serve 12.5 µg a.i. ml^{-1} + *P. aphanidermatum*	2 (8)	4
		Truban 12.5 µg a.i. ml^{-1} + *P. aphanidermatum*	2 (8)	4

[a] Each experiment was repeated at least once and results from 2 repeated experiments are presented in this paper.
[b] Chemicals were applied every 10 days.

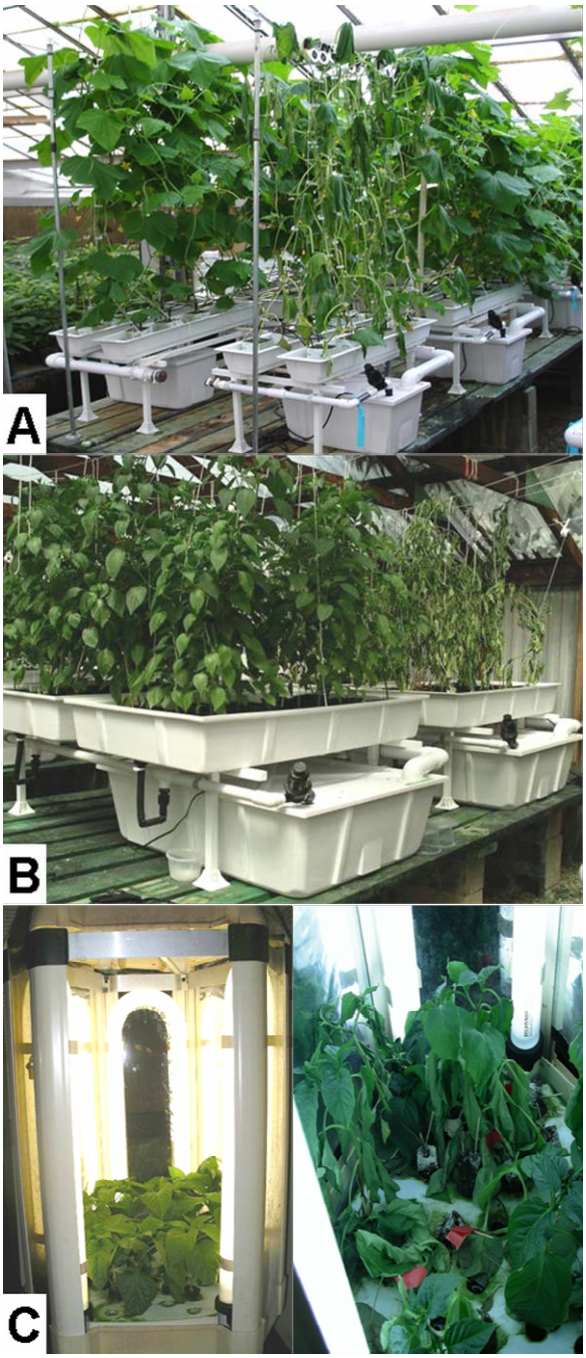

Figure 2.1. Drip-irrigated system: Mortality of cucumber plants after hypocotyl-inoculation of a single plant on one side of a two-sided recirculating hydroponic unit in the absence (right) or presence (left) of a nitrogen stabilizer (A). Ebb-and-Flow system: Mortality of pepper plants after hypocotyl-inoculation of a single plant on one side of a two-sided recirculating hydroponic unit in the absence (right) or presence (left) of a nitrogen stabilizer (B). Floating system: Mortality of pepper plants after zoospore infestation of a single plant per unit in the absence (right) or presence (left) of a nitrogen stabilizer (C).

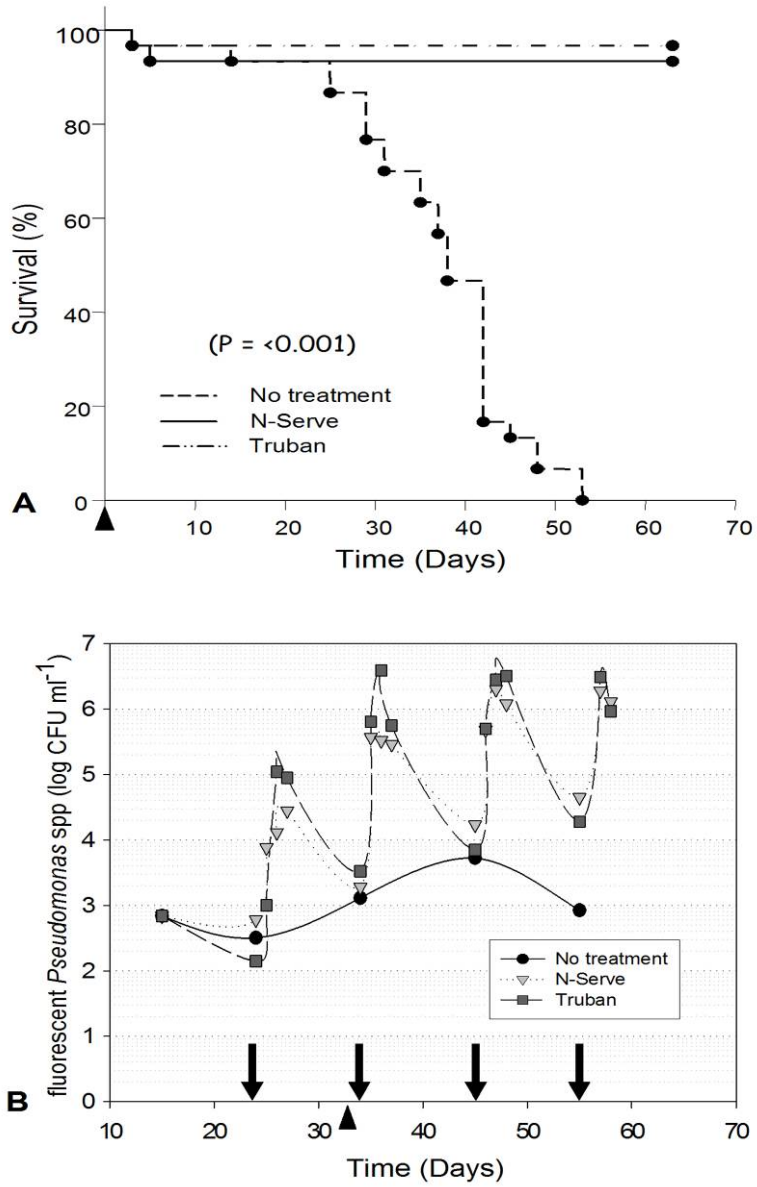

Figure 2.2. Kaplan–Meier estimates of survival functions describing time to death of pepper plants in a ebb and flow hydroponic system after *P. capsici* hypocotyl inoculation. P values were derived with the log-rank test followed by all pairwise multiple comparison procedure (Holm-Sidak method) to isolate groups that differ from each other. Treatments consisted of nutrient solution amended 4 times and not amended with N-Serve® and Truban® (12.5 µg a.i. ml^{-1}), (A). Mean population densities of fluorescent pseudomonads in a ebb and flow hydroponic system with pepper as the host plant. Down arrows indicate the timing of N-Serve® and Truban® applications (12.5 µg a.i. ml^{-1}). Up head arrow indicates *P. capsici* inoculation, (B).

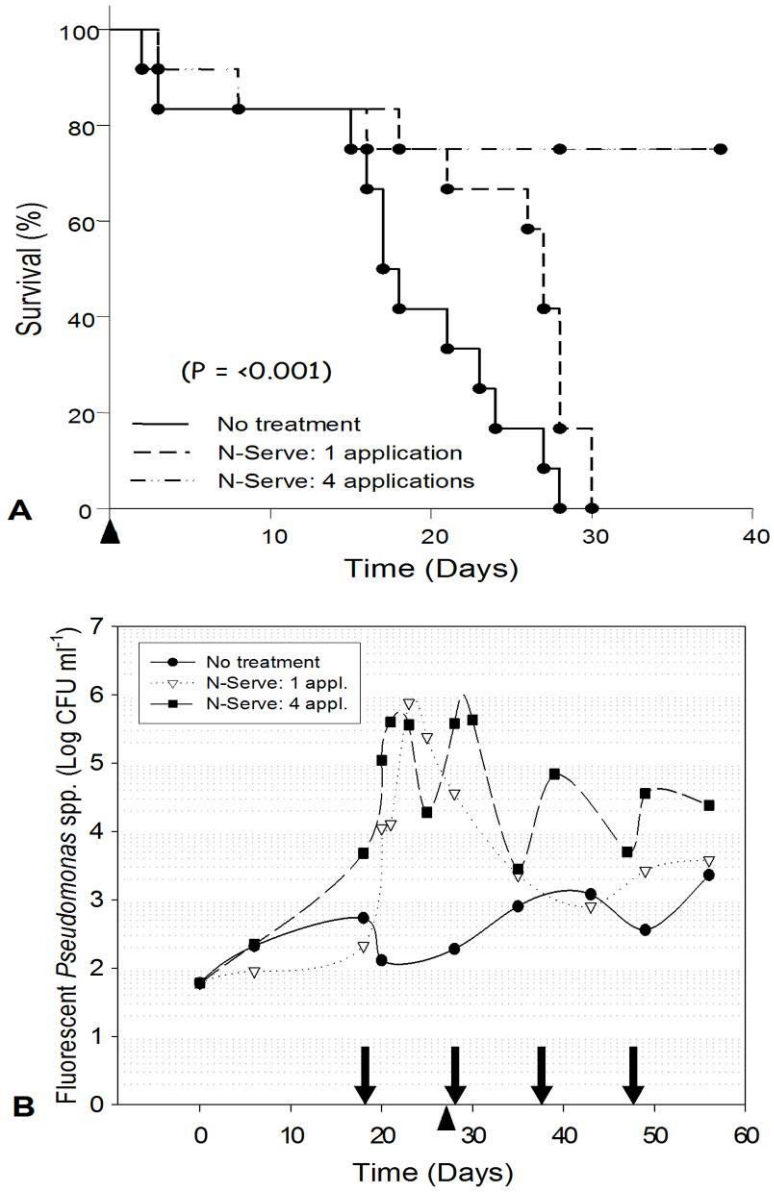

Figure 2.3. Kaplan–Meier estimates of survival functions describing time to death of pepper plants in a drip irrigation hydroponic system after *P. capsici* hypocotyls inoculation. P values were derived with the log-rank test followed by all pairwise multiple comparison procedure (Holm-Sidak method) to isolate groups that differ from each other. Treatments consisted of nutrient solution amended one time, four times and not amended with N-Serve® (12.5 µg a.i. ml^{-1}), (A). Mean population densities of fluorescent Pseudomonads in a drip irrigation hydroponic system with pepper as the host plant. Down arrows indicate the timing of N-Serve® applications (12.5 µg a.i. ml^{-1}). Up head arrow indicates *P. capsici* inoculation, (B).

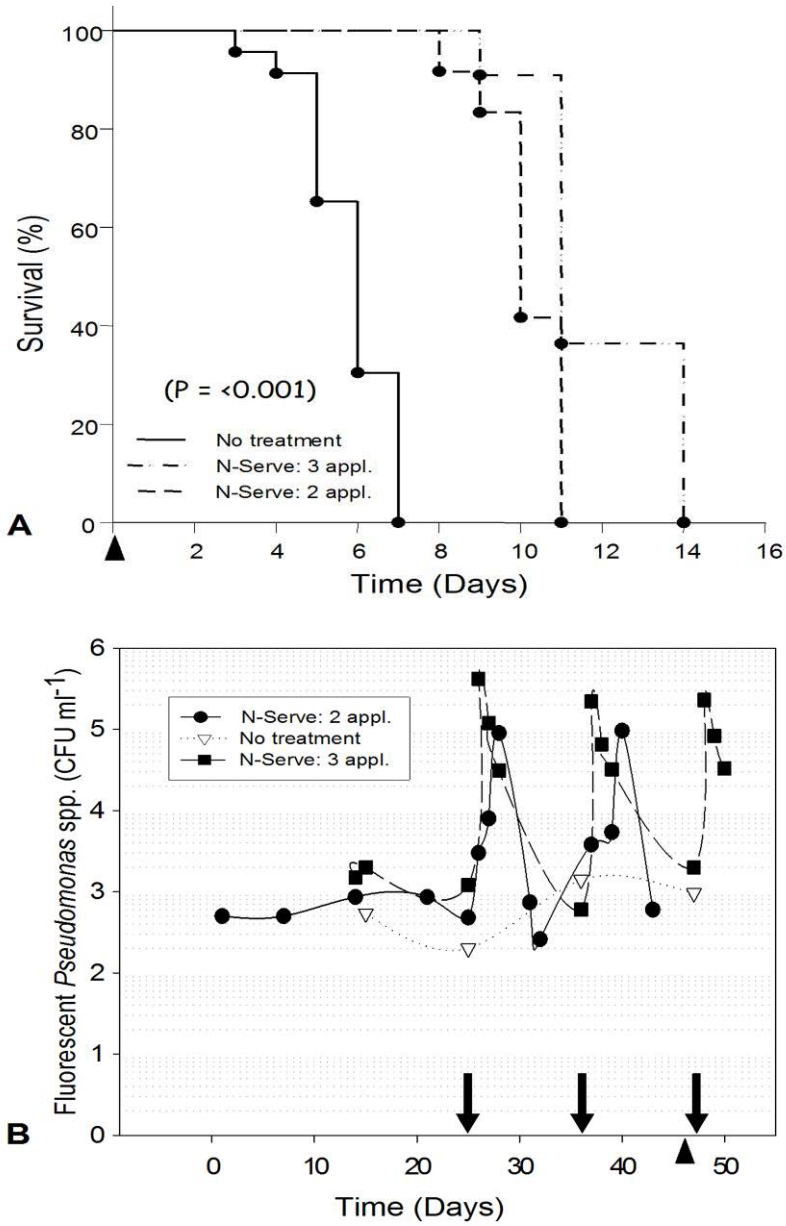

Figure 2.4. Kaplan–Meier estimates of survival functions describing time to death of pepper plants in a floating hydroponic system after *P. capsici* hypocotyl inoculation. P values were derived with the log-rank test followed by all pairwise multiple comparison procedure (Holm-Sidak method) to isolate groups that differ from each other. Treatments consisted of nutrient solution amended two times, three times and not amended with N-Serve® (12.5 µg a.i. ml^{-1}), (A). Mean population densities of fluorescent pseudomonads in a drip irrigation hydroponic system with pepper as the host plant. Down arrows indicate the timing of N-Serve® applications. Up head arrow indicates *P. capsici* inoculation, (B).

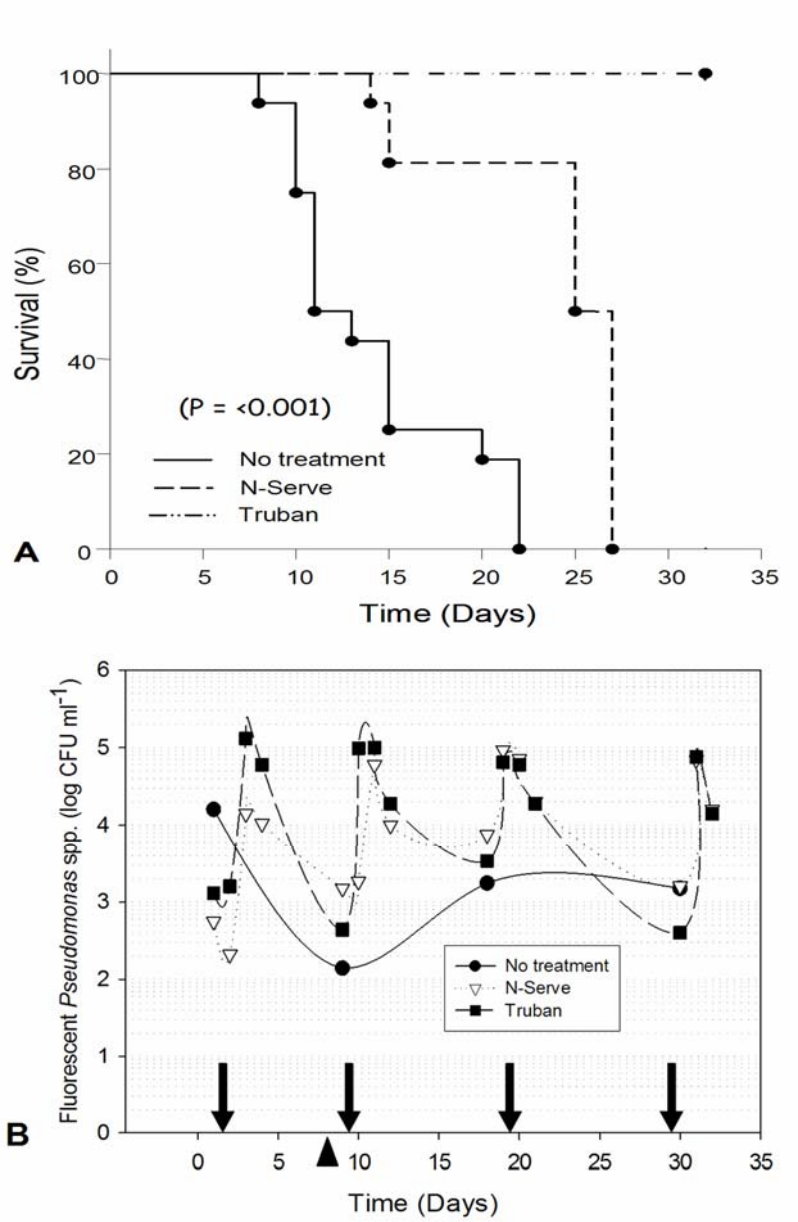

Figure 2.5. Kaplan–Meier estimates of survival functions describing time to death of cucumber plants in a drip irrigation hydroponic system after *P. aphanidermatum* hypocotyl inoculation. P values were derived with the log-rank test followed by all pairwise multiple comparison procedure (Holm-Sidak method) to isolate groups that differ from each other. Treatments consisted of nutrient solution amended 4 times and not amended with N-Serve® and Truban® (12.5 µg a.i. ml^{-1}), (A). Mean population densities of fluorescent pseudomonads in a drip irrigation hydroponic system with cucumber as the host plant. Down arrows indicate the timing of N-Serve® and Truban® applications. Up head arrow indicate *P. capsici* inoculation, (B).

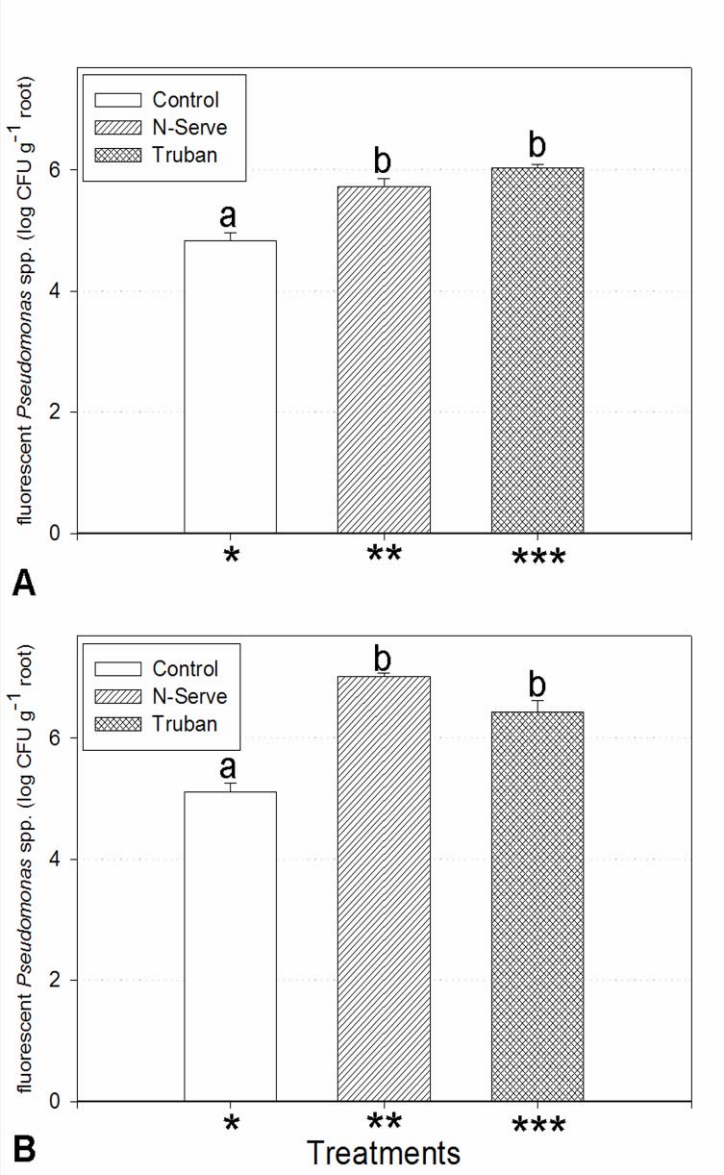

Figure 2.6. Population densities of fluorescent pseudomonads recovered from root of pepper plants growth in a drip irrigation (A) and in an ebb and flow (B) systems, 10 days after the last application of the amendments. Amendments consisted of nutrient solution amended four times and not amended with N-Serve® or Truban® at 12.5 µg a.i. ml^{-1}. Bars with different letters are significantly different at P=0.05 according to the All Pairwise Multiple Comparison Procedures (Student-Newman-Keuls method). Error bars represent the standard error of the mean (n = 4). Population densities of the fluorescent pseudomonads in the nutrient solution were: * 2.8 log CFU ml^{-1} (drip irrigation) and 4.1 log CFU ml^{-1} (ebb and flow) in the un-amended solution; ** 3.7 log CFU ml^{-1} (drip irrigation) and 4.4 log CFU ml^{-1} (ebb and flow) in the N-Serve® treated solution; *** 4 log CFU ml^{-1} (drip irrigation) and 4.7 log CFU ml^{-1} (ebb and flow) in the Truban® treated solution;

Table 2.2. Spearman correlation coefficients (r_s) between the fluorescent pseudomonad population, treatments and disease control (i.e., plant survival time) in different hydroponic irrigation systems with 0, 1, 2, 3 and 4 chemical applications.

Experiment type (# application - Chemical)	Treatments vs. Bacteria r_s ; (P); n	Treatment vs. Disease r_s ; (P); n	Bacteria (CFU ml^{-1}) vs. Disease r_s ; (P); n
Drip Pepper 0, 1 - N-Serve	0.89 (< 0.0001) 24	0.33 (= 0.11) 24	0.31 (= 0.11) 24
Drip Pepper 0, 4 - N-Serve	0.89 (< 0.0001) 24	0.63 (< 0.0001) 24	0.58 (< 0.003) 24
Ebb & Flow Pepper 0, 4 - Truban	0.89 (< 0.0001) 120	0.51 (< 0.0001) 120	0.77 (< 0.0001) 120
Ebb & Flow Pepper 0, 4 - N-Serve	0.89 (< 0.0001) 120	0.52 (< 0.0001) 120	0.78 (< 0.0001) 120
Floating Pepper 0, 2 - N-Serve	0.89 (< 0.0001) 48	0.79 (< 0.0001) 48	0.93 (< 0.0001) 48
Floating Pepper 0, 3 - N-Serve	0.90 (< 0.0001) 46	0.73 (< 0.0001) 46	0.66 (< 0.0001) 46
Drip Cucumber 0, 4 - N-Serve	0.89 (< 0.0001) 64	0.63 (< 0.0001) 64	0.57 (< 0.0001) 64
Drip cucumber 0, 4 - Truban	0.89 (< 0.0001) 64	0.80 (< 0.0001) 64	0.74 (< 0.0001) 64

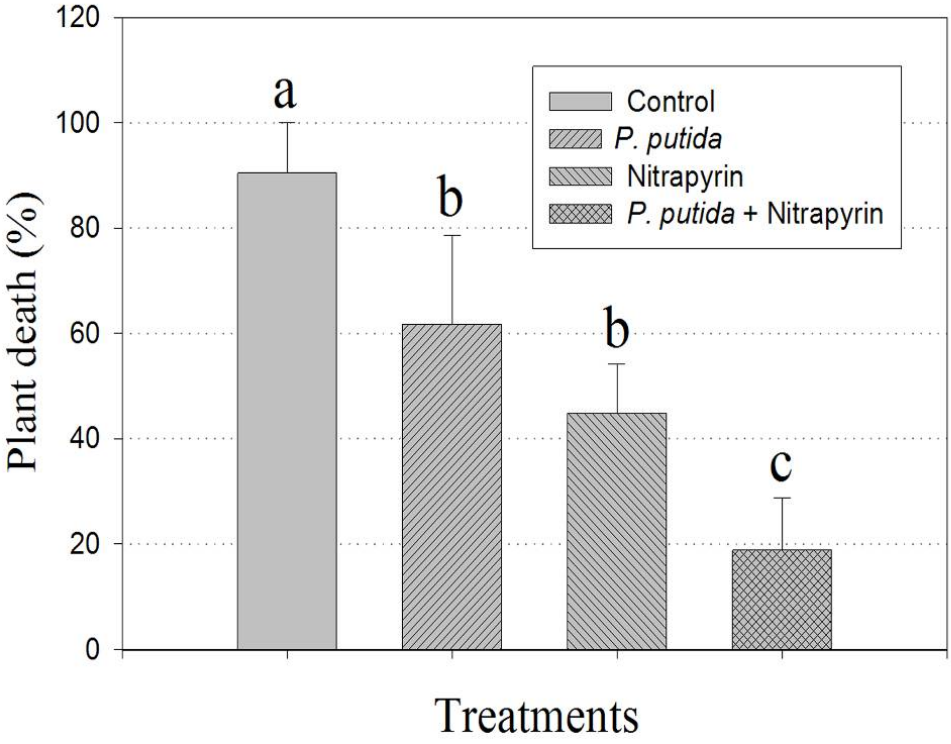

Figure 2.7. Cucumber plant death in a small scales experiment following *Pythium aphanidermatum* inoculation with zoospores. Bars with different letters are significantly different at P=0.05 according to the All Pairwise Multiple Comparison Procedures (Student-Newman-Keuls method). Error bars represent the standard error of the mean (n = 24). Treatments consisted of nutrient solution amended 3 times and not amended with nitrapyrin (12.5 µg a.i. ml^{-1}) and/or *P. putida* pep4 at $5x10^6$ CFU ml^{-1}. Pathogen was introduced one day before the 3rd treatment.

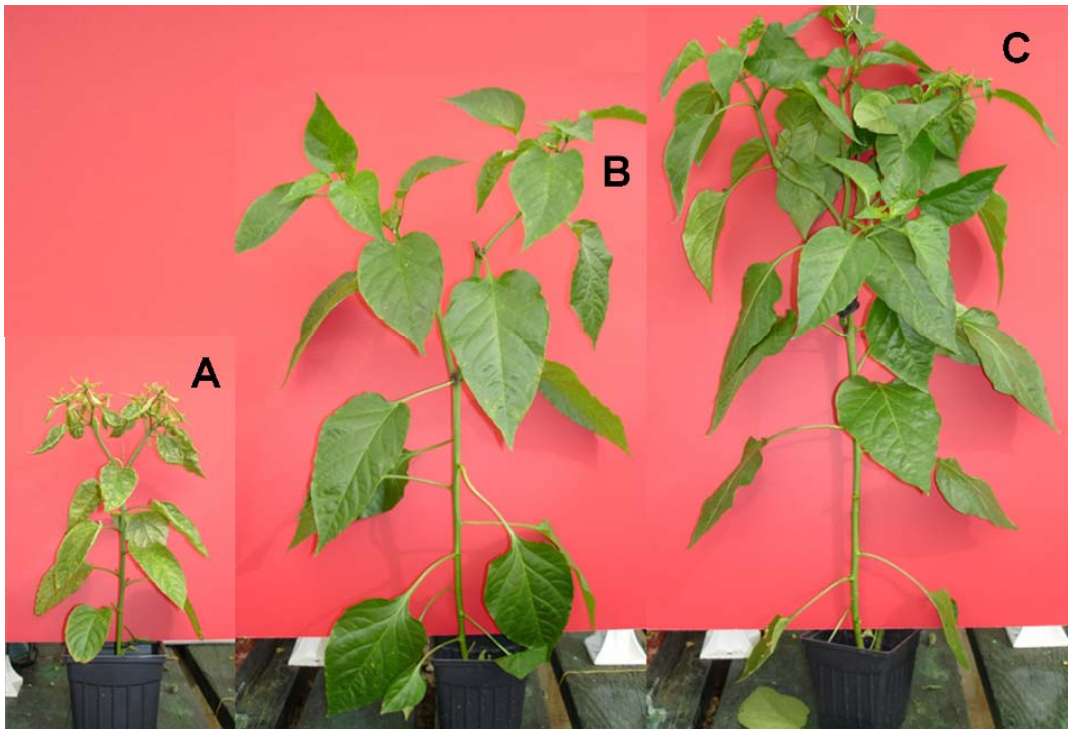

Figure 2.8. Pepper plants grown in recirculating hydroponic unit with 4 application of nitrapyrin at 12.5 µg a.i. ml^{-1}(A); without any nutrient solution amendment (B); and with 4 application of *P. putida* pep4 at $5x10^6$ CFU ml^{-1}(C).

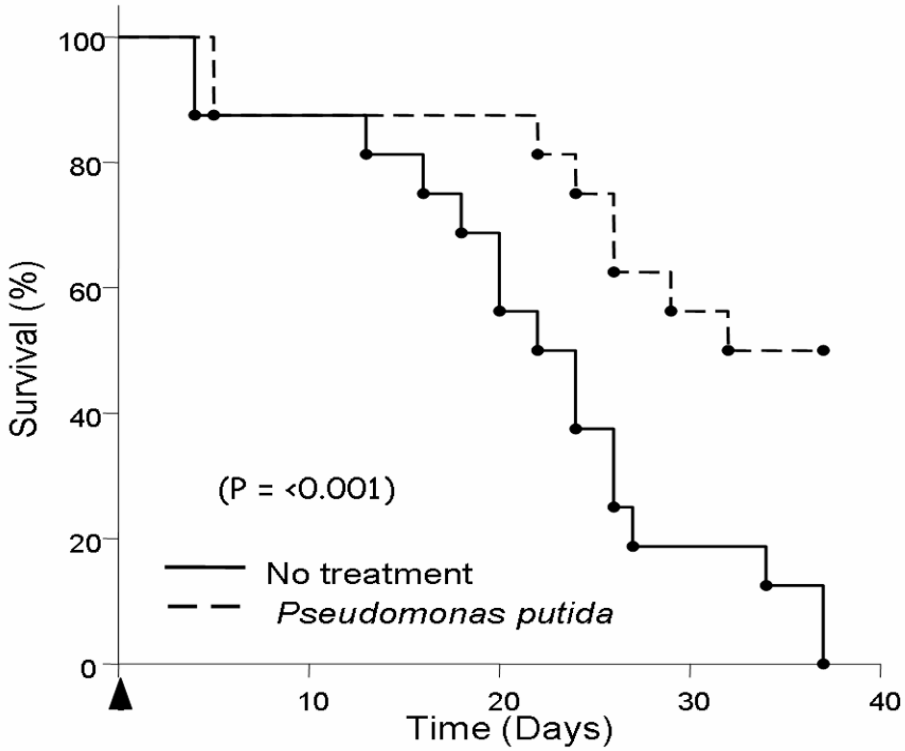

Figure 2.9. Kaplan–Meier estimates of survival functions describing time to death of pepper plants in a drip irrigation hydroponic system after *P. capsici* hypocotyls inoculation. P values were derived with the log-rank test followed by all pairwise multiple comparison procedure (Holm-Sidak method) to isolate groups that differ from each other. Treatments consisted of nutrient solution amended 4 times and not amended with nitrapyrin (12.5 µg a.i. ml^{-1}) and/or *P. putida* pep4 at $5x10^6$ CFU ml^{-1}. Pathogen was introduced one day before the 4^{th} treatment.

Table 2.3. Mycelial growth of *Pythium aphanidermatum* and *Phytophthora capsici* in the presence of nitrapyrin and/or xylene plus 1,2,4-trimethylbenzene or Truban.

Treatments	*P. aphanidermatum*	*P. capsici*
	Mycelium growth after 2 days (%) [a]	Mycelium growth after 7 days (%)
Control	100 [d] a	100 a
Nitrapyrin [b]	54 b	31 c
Xylene + 1,2,4-trimethylbenzene [c]	100 a	94 b
Nitrapyrin + xylene + 1,2,4-trimethylbenzene	47 b[e]	21 c
Truban	19 c	28 c

[a] Cultures were grown on V-8 juice agar and transferred onto V-8 juice agar amended with nitrapyrin and/or xylene plus 1,2,4-trimethylbenzene. Diameters of mycelium growth of individual colonies were measured after un-amended control colonies had reached 100% radial growth on the Petri dishes: two days for *Pythium aphanidermatum* and seven days for *Phytophthora capsici*. Percent growth was calculated dividing the radial growth of each colony by the mean radial growth of the un-amended control colonies.

[b] Mean of four replicates Petri dishes for two repeated experiments.

[c] Nitrapyrin (12.5 µg a.i. ml^{-1}) was applied to 250 ml of sterilized and cooled V8 juice agar (V8C).

[d] Xylene and 1,2,4-trimethylbenzene (20 µg ml^{-1} each) were applied to 250 ml of sterilized and cooled V8 juice agar (V8C)

[e] Value followed by the same letter are not significantly different at P = 0.01.

CHAPTER 3

EFFECT OF NITRIFICATION INHIBITORS, COUPLED WITH THE ADDITION OF KNOWN BIOCONTROL BACTERIUM ON SUPPRESSION OF ROOT ROT OF PEPPERS CAUSED BY *PHYTOPHTHORA CAPSICI* IN A RECIRCULATING HYDROPONIC CULTURAL SYSTEM

Deborah. Pagliaccia, Michael Eugene Stanghellini

(In preparation)

ABSTRACT

Biocontrol bacteria are commonly used as inoculants for improving the control of soil borne plant diseases; however methods for the improvement of their performance are very critical. This study focuses on screening a new strategy for the selective enhancement of effective introduced and indigenous biocontrol strains using specific carbon source amendments (i.e. – N-Serve, Truban and Neem nitrification inhibitors) to the recycled nutrient solution in soilless agriculture.

In laboratory experiments, population densities of Pa 30-84 and Pf-5 increased more than one log unit (from 4.5 log CFU ml^{-1} to 5.8 and 6.13 log CFU ml^{-1}, respectively) in pepper nutrient solution (which contains indigenous bacterial population) following N-Serve amendment. However, data from in vivo experiments revealed superiority of the indigenous microflora over the introduced Pf-5, with a significant Pf5 population decreased following their addition to the nutrient solution in the presence of N-Serve. Indigenous fluorescent pseudomonad populations increase in the nutrient solution and on pepper rhizoplane were also observed in greenhouse experiments following the addition of Truban (synthetic compounds) and Neem (natural compound) nitrification inhibitors to the nutrient solution. Efficacy of the different nutrient solution amendments (i.e. – Neem, Truban and N-Serve) against rot root caused by *P. capsici*, ranged from effective with Truban and N-Serve treatments in both repeated experiments, to marginally effective with Neem oil. The lack of efficacy of Neem oil amendment was attributed to the inability of the treatment (both low concentration of the active ingredient and inadequate fluorescent pseudomonad performance) to stop the spread of zoospore propagules in the nutrient solution as proved by the results from the baiting essays. On the contrary, N-Serve and Truban consistently decrease the zoospores presence, thus their spread in the recirculating nutrient solution, which would explain the disease control improvement obtained in the present and in our previous studies.

It was concluded that for the selective enhancement of biocontrol in recirculating nutrient solution, indigenous bacteria are more suitable than introduced strain and that specific carbon source such as naturals and synthetics nitrification inhibitors have potential for the development of new integrated disease management strategies.

INTRODUCTION

One of the challenges to improving biological control of soil borne plant diseases is to overcome the inconsistencies in performance attributed in part to the effects of competing microflora and to the inability of the biocontrol bacteria to maintain critical threshold populations necessary for sustained biocontrol activity. Alteration of the environment to favor the biological control organism and disfavor competitive microflora appears to be necessary to enhance and prolong the desired responses [4, 5].

Data collected over 3 years from in vitro experiments and greenhouse trials carried out with hydroponic systems have shown that the application of specific carbon source (N-Serve and Truban) enhance the population densities of indigenous fluorescent pseudomonad in the nutrient solution and on plant roots [15, 16, 17, 18]. We also demonstrated the efficacy of such chemical amendments to the recycled nutrient solution in the management of root disease of pepper and cucumber caused by *Phytophthora capsici* and *Pythium aphanidermatum,* respectively. Our studies suggested that both the active and inert ingredients in N-Serve® were involved in disease suppression, and serve (directly or indirectly) as biological and chemical control at the same time, since they exhibited bi-functional properties [15, 18].

With the present study we investigated such bi-functional properties for the development of new ways to control plant diseases with a minimum use of pesticides by enhancing biologically-based pest management strategies.

Several studies have demonstrated that many of the antibiotics produced by introduced biocontrol agents play a key role in the suppression of various soilborne plant pathogens [22] and that their biosynthesis is regulated by quorum sensing molecules (i.e. - N-acyl homoserine lactones - AHL), which are produced when the bacterial population reaches high densities [6, 7, 11, 20]. Therefore, our first objective was to investigate the possibility of a selective enhancement of well known biocontrol strains such as *Pseudomonas aureofaciens* Pa 30-84 [6, 21, 31] and *Pseudomonas fluorescent* Pf5 [19, 24] in the recirculating nutrient solution using N-Serve® amendment. *In vitro* studies were first performed with both Pa 30-84 and Pf5 and then Pf5 was chosen for the greenhouse experiments. Pf5 population dynamics was monitored in the nutrient solution during the greenhouse experiments and rhizoplane colonization assessed at the end of the experiment.

The second objective of the present study was to perform experiments to investigate the population dynamics of indigenous fluorescent pseudomonad population following the amendment of a different nitrification inhibitor, Neem oil, to the recirculating nutrient solution of pepper hydroponic cultural systems. Although much of the activity of certain biological product (i.e. plant extracts and oil) is due to the chemical active ingredients in the material, the other components in the formulation of the oil or extract may have different effect on the pathogen as well as the indigenous microflora. Neem oil is a vegetable oil pressed from the fruits and seeds of Neem (*Azadirachta indica*) [2, 29]. The active compound in neem oil consists of various terpenes/ triterpenes (azadirachtin, epinimbin, nimbin, deacetylnimbin, salanin, deacetylsalanin) which had been previously reported to exert a nitrification-inhibiting effect on *Nitrosomonas* spp. [1]. Long fatty acids and glycerides, which could act as a carbon source for bacterial growth, are the other components contained in Neem. Changes in the fluorescent pseudomonad population in the recirculating nutrient solution following Neem application was assessed and compared to N-Serve and Truban amendments, along with the establishment of population of the fluorescent Pseudomonad in the rhizoplane of pepper plants.

Finally the last objective was to evaluate these amendments on their efficacy in reducing root disease of pepper caused by *Phytophthora capsici* and to monitor the presence of the pathogen, most likely as zoospores, in the recirculating nutrient solution after pathogen infestation.

MATERIALS AND METHODS

***In vitro* effect of N-Serve® on the biocontrol isolates Pf5 and Pa 30-84 in nutrient solution in presence of indigenous fluorescent pseudomonads.** To study the effect of N-Serve® on the growth of the known biocontrol bacteria, i.e., *Pseudomonas fluorescent* Pf5 and *Pseudomonas aureofaciens* Pa 30-84, in the presence of competition from other indigenous bacteria, the isolates was added to samples of nutrient solution. *P. aureofaciens* 30-84 is a wild type strain producing the antibiotic phenazine while *P. fluorescence* Pf5, also a wild type isolate, produces these antibiotics, i.e., 2,4-diacetylphloroglucinol (2,4 DAPG), pyrrolnitrin and pyoluteorin.

Nutrient solution samples were collected from a non-treated reservoir of a hydroponic unit from a pepper experiment. Treatments consisted of 0.5L of nutrient solution (containing indigenous bacteria) seeded with Pf5 rif^{+} or Pa 30-84 rif^{+} (approximately 4.5 log CFU ml^{-1}) amended with N-Serve® at 12.5 µg a.i. ml^{-1} and a control treatment of nutrient solution with Pf5 rif^{+} or Pa 30-84 rif^{+} not amended with N-Serve®. The treatments were incubated at 28 °C with continuous agitation on a rotary shaker (100 rpm). Population of the indigenous fluorescent pseudomonads, the introduced Pf5 rif^{+} and Pa 30-84 rif^{+} strains, were monitored 1, 3, 5 and 12 days after adding the treatment, collecting ten ml of nutrient solution from each treatment. Fifty ul of different dilutions of the sample were plated in triplicate, using a spiral plater (Autoplate 4000 – Spiral Biotech, Inc), onto 10 cm diameter Petri dishes containing King's B medium (KB). For enumeration of the total fluorescent pseudomonad population (which included both the indigenous fluorescent population and introduced Pf5 or Pa 30-84) the KB agar was supplemented with 50 µg ml^{-1} cycloheximide (Calbiochen) and 75 µg ml^{-1} penicillin (Calbiochen). For the enumeration of the introduced Pf5 rif^{+} and Pa 30-84 rif^{+} isolates, the KB agar was supplemented with rifampicin at100 µg ml^{-1}. Colonies were counted under UV light after 24 h of incubation at 28° C.

Inocula of Pf5-rif^{+} and Pa 30-84-rif^{+} were obtained by growing the isolates in Luria broth (LB) (EMD Chemicals Inc.) containing rifampicin at 100 µg ml^{-1} at 28°C in a shaker (120 rpm) for 24 h, then in KB agar containing rifampicin 100 µg ml^{-1} for 48 h. Finally bacteria were harvested from the KB agar into 50 ml tubes of 0.01 M $MgSO_4$ solution. Cells were washed twice (pelleted by centrifugation) in $MgSO_4$ and re-suspended in sterile distilled water. To obtain 10^8 CFU ml^{-1}, cells densities (OD=0.25) were assessed at 600 nm using Genesys 10vs Spectrophotometer (Spectronic Unicam). These suspensions were then diluted to the 0.5L flasks with the nutrient solution to obtain the desired population densities for the study.

Greenhouse study

Hosts and pathogens. All experiments were conducted using pepper (*Capsicum annuum* L. cv. Joe Parker) as the host and *Phytophthora capsici* L. as the root pathogen. Plant survival rates were assessed after inoculation with *Phytophthora capsici* L. Stock cultures

of *Phytophthora capsici* L. were stored in sterile distilled water, and working cultures were grown on 20% clarified V8 juice agar (V8C).

Hydroponic systems. Experiments were conducted in temperature-controlled greenhouses (20-32°C) (Fig. 3.1A) using a drip irrigation system (Jetstream™, American Agritech). Drip irrigation systems consisting of recirculating hydroponic units with an upper container, for plant growth, and a lower reservoir where the nutrient solution is kept and recycled. Plants were top irrigated with nutrient solution, via drip tubing outfitted with a 7.2-L/h emitter, for 30 minutes, 3 to 5 times during a 24-h period (3h interval - 8 a.m. and 8 p.m.) depending of the plant growth stage. Unless otherwise specified, all cultural practices were the same as those employed in previous investigations [14, 28].

Pepper seeds were sterilized in a NaClO solution (1%, v/v) for 5 minutes, washed with ethanol, then with sterile distilled water and placed to germinate on Oasis Horticubes (Smithers-Oasis, Kent, OH) in a temperature-controlled incubator at 25°C. Three-week-old seedlings were then transplanted into a commercial organic (peat-based) potting mix (Supersoil®, Rod McLellan Company, San Mateo, CA) in 11 cm×11 cm×12 cm plastic pots. Eight potted plants (at 4-leaf stage) were placed in each unit of the drip irrigation system, with 12 cm between plants.

Treatments. There were two experiments in which the reservoirs were treated with four different amendments, and each experiment lasted for 20 weeks. In each experiment, there were up to six treatments with 3 replications per treatment (except the control that had 2 replications) and 8 plants per replication. The number of treatments and treatment replications per experiment are summarized in Table 3.1. Three chemicals were used as amendments to the recirculating nutrient solutions: N-Serve® 24 (Nitrapyrin a.i., Dow AgroSciences, Indianapolis), Truban®25E (Etridiazole a.i., The Scotts Company), and Neem oil KillerB (Azadirachtin a.i. 0.15% EC).

N-Serve®24 is a commercial nitrification inhibitor containing 22.2% nitrapyrin, 2.25% related chlorinated pyridines, and 75.55% inert ingredients (23% 1,2,4-trimethylbenzene and 2% xylene-mixed isomers, are the primary inert ingredients). Truban®25E (Etridiazole a.i., The Scotts Company) is a contact fungicide for the control of

damping-off, root and stem rot disease in ornamental and nursery crops. Etridiazole, the active ingredient of Truban®25E, acts also as nitrification inhibitor and it is marketed under the trade names Dwell® or Terrazole® (Uniroyal Chemical Company). Truban® 25EC contains 25% etridiazole and 75% inert ingredients (15% xylene-mixed isomers, is the primary inert ingredients). Neem oil KillerB is an insecticide, extracted from neem tree seed, containing Azadirachtin (0.15% EC) as active ingredient and other constituent such long fatty acid and glycerides.

To investigate the possibility of a selective enhancement of a known introduced biocontrol strain, in addition to the N-Serve® treatments by itself, these experiments also included nutrient solution treated with N-Serve® and in the presence of *Pseudomonas fluorescent* Pf5 Rif^+ at 4-4.5 log CFU ml^{-1}. A rifampicin-resistant strain of bacterium was obtain as described previously and then appropriate amounts diluted to the nutrient solution in the reservoir to obtain the desired population densities for the study.

All treatments were applied 1h before the start of the irrigation at 10 days intervals for a total of 5 applications. N-Serve®, Truban® and Neem oil treatments were added directly to the nutrient solution. Biocontrol Pf5 was added to the extra N-Serve treatment just after the chemical amendment to have a final bacterial concentration of 4-4.5 log CFU ml^{-1}.

Pathogen inoculation technique. Pathogen infestation of an individual hydroponic unit (Figure 1), which occurred between the second and the third chemical application, was achieved by hypocotyl inoculation of a single plant from each system. This method of infestation permitted the evaluation of pathogen spread within a unit following colonization and natural reproduction of the pathogen on the roots of the inoculated plants. A 10-mm-diameter disk, cut from the advancing margin of a 5-day-old V8 agar culture of *P. capsici* was placed in contact with the lower hypocotyls (immediately below the substrate surface) of one pepper plant per recirculating system.

Monitoring indigenous and introduced fluorescent pseudomonad populations in the nutrient solution and bacterial identification. Twenty ml samples of the nutrient solution were collected at different time intervals (primarly taken at 0, 24, 48 and 72 hours after chemical amendment) from the reservoir of each treatment. After serial 10-fold dilutions, aliquots were plated in triplicate, onto 10 cm diameter Petri dishes containing

King's B medium (KB), as described previously, for enumeration of the indigenous fluorescent pseudomonads and the introduced Pf5 rif^{+} isolate.

Dominant fluorescent pseudomonads isolates, growing on tryptic soy agar (TSA) medium, originated from the N-Serve® (10 isolates), Truban® (10 isolates) and Neem oil (6 isolates) amended nutrient solutions from the greenhouses experiments, were compared with those obtained in previous studies [16]. Petri dishes containing 50–100 single colonies were chosen and colonies were randomly selected from a different sector of each plate. The isolated colonies were purified by repeated streaking onto King B agar. Purified isolates were stored at room temperature on KB agar plates or maintained for long-term storage at –70 °C in nutrient broth (Difco Laboratories) supplemented with 15% glycerol. A total of twenty-six bacterial isolates were identified using the BIOLOG™ GN plates and the Biolog Microlog2 4.20 software database (Biolog, Inc, Hayward, CA).

Monitoring indigenous and introduced fluorescent pseudomonad populations in the rhizoplane. The effects of N-Serve®, Truban, and Neem amendments to the nutrient solution on the pepper rhizoplane population of fluorescent pseudomonads (i.e. – root colonization) were investigated and compared to rhizoplane fluorescent pseudomonad populations in the un-amended treatments. Ten days after the last application of the amendments, two plants were randomly chosen from each treatment. The roots were excised, blotted to remove excess water and weighed. The roots, 0.5 g, were then placed in 10 ml sterile distilled water and agitated in a shaker (200 rpm) for 1 h at 28°C. After serial 10-fold dilutions, aliquots of the suspension were plated, in triplicates, onto King's B medium (KB) as described above for enumeration of the fluorescent pseudomonads. Additionally, for the detection and enumeration of the introduced Pf5 rif^{+} isolate on the roots, aliquots of the suspension from N-Serve® plus Pf5 rif^{+} treatment were plated on King's B medium (KB) plates supplemented with rifampicin (100 μg ml^{-1}). The experiment was repeated twice.

Survival of plants in the absence or presence of N-Serve®, Truban®, Neem and the *Pseudomonas fluorescent* Pf5 rif^{+}. Disease incidence (i.e. pepper plant mortality after pathogen introduction) was monitored daily throughout the duration of each experiment. The data set of this study contained, however, many censored observations (i.e., plants that

have not died by the end of the assessment period); therefore time to death, the dependent variable, was interpreted as a "length of survival", and survival analysis techniques were used to describe and model the data. The application of survival analysis has been limited in plant pathology. In the last few years however, this model have been successfully employed by other researchers [9, 25, 26]. This survival analysis technique has been used also in our previous studies [15].

Pathogen recovery - monitoring zoospore presence and root infection. Presence of the pathogen, most likely as zoospores, in the recirculating nutrient solution was monitored using a baiting assay. Ten days after pathogen infestation, at 10 days interval and for three consecutive times, 300 ml of nutrient solution was collected in a 500-ml beaker from each reservoir, thirty minutes after cessation of the afternoon irrigation. Twenty 0.5-cm diameter disks of fresh pepper leaves were floated on the surface of the samples. After 24 h, the disks were plated onto PARPH, a *Phytophthora* selective medium [8]. After 72 h incubation at 28° C, disks were observed microscopically (30× magnification) for the presence of mycelium and sporangia along the margin and the number of baits that were colonized was recorded.

Root infection was assessed at the end of each experiment. Five root segments, each 1-2 cm long, were excised from three plants from each treatment and plated onto PARPH. After 72 h incubation at 28° C, hyphae emerging from the roots were transferred to clarified V8 agar medium, and *P. capsici* cultures were identified.

Statistical analysis. Greenhouse experiments were conducted using a randomized complete block design with three blocks and one replicated unit (containing eight plants of every treatment) per block, except the control that had 2 replications.

Plant death from root rot (caused by *P. capsici* or *P. aphanidermatum*) was the primary end point and, for every treatment, was assessed by plotting survival curves according to the Kaplan-Meier method [25]. The data were analyzed with a closeout (study censor) date opted to be 10 days after the last plant had died in the *P. capsici* treatment. Data were considered censored when plant had not died by the end of the closeout date. In each experiment carried out, the differences between the survival curves were tested for statistical significance with the log-rank test followed by a multiple comparison procedure

(Holm-Sidak method) to isolate the treatments that differ from the others. Homogeneity test of data from the two repeated experiments was evaluated by Cox Proportional Hazard model using a Breslow test at P=0.05. Since the data, from the separate trials differ significantly, they were not pooled for the Kaplan-Meier analysis.

Data from the fluorescent pseudomonad root colonization study in the greenhouse were found to be non-normal; therefore data were analyzed by the Kruskal-Wallis One Way Analysis of Variance on rank, followed by the All Pairwise Multiple Comparison Procedures (Student-Newman-Keuls Method). Fluorescent pseudomonad population data were $\log_{10}$ transformed before statistical analysis.

For the baiting assay, percent recovery data were normalized using the arcsine transformation (the untransformed values are presented for ease of interpretation) and analyzed by the One Way Analysis of Variance, followed by the All Pairwise Multiple Comparison Procedures (Holm-Sidak method). If also the transformed data result not normal distributed, were analyzed by the Kruskal-Wallis One Way Analysis of Variance on rank, followed by the All Pairwise Multiple Comparison Procedures (Student-Newman-Keuls Method).

All data were analyzed using SigmaStat 3.0 statistical software package (SPSS Science, Chicago, IL). All experiments were repeated at least once.

RESULTS

***In vitro* effect of N-Serve® on the biocontrols isolates Pf5 rif$^+$ and Pa 30-84 rif$^+$ in nutrient solution in presence of indigenous fluorescent pseudomonads.** Under growth chamber conditions, population densities of *Pseudomonas aureofaciens* 30-84 and *Pseudomonas fluorescens* Pf-5 increased in pepper nutrient solution (which contains indigenous bacterial population) following N-Serve amendment (Fig. 3.2A and 3.2B). The initial population of Pa 30-84 rif$^+$ and Pf5 rif$^+$ at time 0 was approximately 4.5 log CFU ml^{-1} of nutrient solution and 4.3 to 4.5 log CFU ml^{-1} for the indigenous fluorescent *Pseudomonas* spp. population. After 72h following N-Serve® treatment at 25 µg a.i. ml^{-1}, Pa 30-84 rif$^+$ and Pf5 rif$^+$ populations increased by one log unit (from 4.5 log CFU ml^{-1} to 5.8 and 6.13

log CFU ml^{-1}, respectively) while a decline occurred in the non-amended control treatment (from 4.5 log CFU ml^{-1} to 3.87 and 4.04 log CFU ml^{-1}).

Increases in population densities also occurred in the total (i.e. the sum of introduced and indigenous) *Pseudomonas* spp. population (6 and 6.48 log CFU ml^{-1}) in the N-Serve® treated solution 3 days after amendment compare to decrease in the control treatment ($3x10^4$ and $1.1x10^4$ CFU ml^{-1}, respectively) (Figure 3.2A and 3.2B). The indigenous *Pseudomonas* spp. populations were not affected by the introduced population Pa 30-84 rif^+ and Pf5 rif^+ either in the N-Serve amended or non-amended nutrient solution, thus recovered twice as many as many as the introduced Pa 30-84 rif^+ and Pf5 rif^+.

Greenhouse study

Monitoring indigenous and introduced fluorescent pseudomonad populations in the nutrient solution and bacterial identification. The initial population densities of Pf5 rif^+ after introduction to the nutrient solution was 4-4.5 log CFU ml^{-1}. The addition of N-Serve at 12.5 µg a.i. ml^{-1} did not increase Pf5 rif^+ population densities. On the contrary, a rapid decrease of the Pf5 rif^+ population to undetectable levels was observed after each consecutive Pf5 rif^+ amendment, with a single exemption (Fig. 3.3). In contrast, the total population of fluorescent pseudomonads (which included both the indigenous fluorescent population and Pf5) increased from 3-4 log CFU ml^-1 to 5-6 log CFU ml^{-1}, following the addition of N-Serve to the recirculating nutrient solution (Fig. 3.3).

The population dynamics of the indigenous fluorescent *Pseudomonas* spp. in recirculating nutrient solutions amendment with N-Serve®, Truban® and Neem oil were monitored in the hydroponic systems used in the cultivation of pepper (Fig. 3.4A and 3.4B). In two separate experiments, greater bacterial population increases were consistently recorded in all amended nutrient solutions compared to non-amended nutrient solutions. Bacterial population increases, which were two or more order of magnitude higher than their respective controls, occurred within 48 to 72 h after each consecutive addition of N-Serve®, Truban® or Neem oil to the nutrient solution (Fig. 3.4A and 3.4B).

Bacterial identification. All 26 isolates of dominant bacteria, randomly selected (on TSA medium) from different treated nutrient solutions were fluorescent pseudomonads. All 10 isolates of dominant bacteria, randomly selected (on TSA medium) from N-Serve® and Truban amended nutrient solutions, were identified using Biolog™, with similarity

indices of 70% and 73% (ranging from 60 to 91%) respectively, as *Pseudomonas putida* biotype A. 50% of Neem isolates (3/6) were identified, with similarity indices of 60%, as *Pseudomonas putida* biotype A, and the other 50% were identified, with similarity indices of 67%, as *Pseudomonas maculicola.*

Monitoring indigenous and introduced fluorescent pseudomonad populations in the rhizoplane. Fluorescent pseudomonad populations (CFU g^{-1} of roots) were significantly higher (P=<0.001) in Neem, N-Serve, and Truban treated nutrient solution compared to roots from the un-amended solution. However, we noted that the fluorescent pseudomonads recovered from roots from systems amended with N-Serve® and Truban exhibited greater fluorescence than the fluorescent pseudomonads from Neem treated and the un-amended systems when grown on KB medium (Fig. 3.6).

The fluorescence produced on KB medium under UV light at 600 nm, which indicates siderophores production, from pseudomonad recovered on the roots from Serve® treatment along with Pf5 amendment was compared to Neem treatment and the un-amended systems. Pseudomonad from Serve® treatment along with Pf5 rif^{+}, even though showed a higher fluorescence compare to Neem amended and the un-amended systems, did not have a significant higher total fluorescent pseudomonads population (i.e. the sum of introduced and indigenous). Additionally, population of the introduced Pf5 rif^{+} could not be recovered on the root.

Survival of plants in the absence or presence of N-Serve®, Truban®, Neem and the *Pseudomonas fluorescent* Pf5 rif^{+}. All hypocotyl-inoculated pepper plants, with a single exception for three Truban treated pepper plant, in all greenhouse experiments and all treatments wilted and died within 2 weeks after inoculation (Fig. 3.7A, 3.7B). In the absence of N-Serve®, Truban®, Neem and N-Serve® plus Pf5 rif^{+}, all non-inoculated pepper plants died within the next 32 days in the first experiment and 44 days in the second experiment. No plants died in the non-inoculated treatments.

Figures 3.7A and 3.7B show the Kaplan-Meier estimates of the probability of death from root rot in the experiments with and without treatments.

Treatments with Truban® significantly increased ($P=1.3x10^{-12}$ in the 1st experiment and $P=7.8x10^{-10}$ in the 2nd experiment) pepper plant survival compared to the control.

Specifically, among pepper plants treated with Truban® the risk of root rot caused by *P. capsici* declined within 30 days after inoculation to about 5 to 10% (the only dead plants were the inoculated ones) with no progress in plant death over the 42 and 54 days trials, for the first and second experiment, respectively.

Among pepper plants treated with N-Serve®, plant survival was significantly greater, 63% ($P<0.0001$ in the 1st experiment) and 83% ($P<0.0001$ in the 2nd experiment), 32 days after pathogen inoculation compare to the control (0 % in both experiments). However a better survival (83%) over the 42 days trials was observed in the 2nd experiment compare to the 25% survival over the 54 days trials in the 1st experiment.

Pf5 amendment at 4-4.5 log CFU ml^{-1} to the nutrient solution along with Serve® induced a significant ($P=1.2x10^{-4}$ in the 1st experiment and $P=2.7x10^{-8}$ in the 2nd experiment) longer pepper survival time compare to the control, but did not show a significant survival improvement ($P=0.11$ in the 1st experiment and $P=0.69$ in the 2nd experiment) compared to the Serve® treatment alone.

With Neem oil treatment, the Kaplan-Meier survival curves in both experiments are displaced toward shorter survival compared to all the others treatments with plant loss occurring earlier after pathogen inoculation. Neem amended treatments had a significant better survival compare to the control only in the second experiment ($P=8.7x10^{-5}$), where plant death reached 75% at the end of the experiment. No significant difference ($P=0.27$) was observed between Neem amended treatments and the control in the first experiment.

Pathogen recovery - monitoring zoospore presence. During the two pepper experiments a baiting assay was used to recovery zoospores from the nutrient solution (expressed in % of pepper foliar bait colonized by *P. capsici* zoospores), after *P. capsici* hypocotyls inoculation (Table 3.2). *P. capsici* was not recovered from any non-infested treatments. Ten days after *P. capsici* infestation (1st baiting assay), no significant difference ($P=0.46$) was observed between treatments (2nd experiment). At the 2nd bait assay (20 days after *P. capsici* infestation) propagules started to be recovered in highest number in both experiments, although with a significant difference beetwen treatments only in the 1st experiment ($P=0.046$). Specifically, in the 1st experiment infected bait leaves increased to 68% in the un-amended nutrient solution and 78% in the Neem treated solution, while Truban, N-Serve and N-Serve plus Pf5 recovery was significantly reduced to 0, 18 and

23%, respectively. In the 2nd experiment (2nd bait), untreated nutrient and Neem treatments also had the highest number of infected bait leaves; with no significant difference between treatments (P=0.46). At the 3rd bait assay, low numbers of *P. capsici* infected baits leaves were recovered with N-Serve and Truban (10 and 0%) in the 1st experiment with a high significant difference between all treatment (P=0.018). A lower baits infection was observed in the 2nd experiment with no significant difference beetwen treatments (P=0.51), although the untreated nutrient solution reached 25% of baits infection, compared to the 0% of Truban, 2% of N-Serve and 7% of Neem and N-Serve plus Pf5 treatments.

DISCUSSION

In vitro studies (Fig. 3.2) showed that great population increases of known biocontrol agents, i.e., - Pf-5 and Pa 30-84, occurred following their addition to the nutrient solution in the presence of N-Serve. Although their population increases were lower (2X) than the population increases of the indigenous fluorescent pseudomonad, the concomitant bacterial population enhancement, suggested that Pf-5 and Pa 30-84 were ecologically fit and competitive in the nutrient solution versus the indigenous population which could improve biological control. However, an examination of the data from in vivo studies (Fig. 3.3) revealed that Pf-5 was not competitive with indigenous fluorescent pseudomonads in greenhouse studies and their populations decreased significantly following their addition to the nutrient solution in the presence of N-Serve. The inability of Pf-5 to compete with the indigenous fluorescent pseudomonads was substantiated by their absence on roots (Fig. 3.6). However, Pf-5 isolate caused reduction in the population of indigenous fluorescent pseudomonad in Pf-5-treated solution, compared with N-Serve treatment alone.

These results indicates that although it is important to be able to increase the population density of an introduced biocontrol organism such as a Pa30-84 and Pf5, it is perhaps more advantageous to focus on enhancing the indigenous fluorescent pseudomonad population, which are metabolically versatile and capable of utilizing many natural and xenobiotic compounds [10, 13]. In our system, *Pseudomonas putida* metabolic versatility together with the ubiquity and the adaptability, showed to give an advantage to prolong over longer periods their beneficial activity as biocontrol [15,16].

Additionally, the concept of selectively enhancing the population of an indigenous bacterium by the addition of a carbon source was extended also to natural product. Our

results from the greenhouse experiments indicate that Neem oil, like N-Serve and Truban, selectively and significantly enhanced the fluorescent pseudomonad population (*P. putida* and *P. maculicola* strains) in the recirculating nutrient solution reservoirs of hydroponically-grown pepper plants (Fig. 3.4A and Fig. 3.4B) and did not cause phytotoxicity on plants. Our results support and extend the concept of selective enhancement of a specific microorganism in a root/soil microbial communities by providing an exotic substrate, in this case a natural product such Neem oil. In the present study we did not investigate the distinct role of the active vs. inert ingredients in Neem oil in the bacterial enhancement but we assumed that the Neem constituents, i.e., - long fatty acids and glycerides, would serve as carbon source to the indigenous fluorescent pseudomonad population while, the active ingredient, i.e., - azadirachtin, could function as fungicide as shown in previous studies [3, 23, 27]. Moreover, the presence of high populations of fluorescent pseudomonads on roots (Fig. 3.6) following Neem application provides evidence of the ability of the enhanced fluorescent pseudomonads in the nutrient solution to colonize roots. However, compared to the pseudomonads isolated from N-Serve and Truban amendments, pseudomonad isolates recovered from Neem-amended solutions seemed to produce lower levels of siderophores as shown by the weak brightness of the fluorescence (Fig. 3.5) on the KB medium. Siderophore production in the rhizosphere and soil by fluorescent pseudomonads has repeatedly shown to play a role in disease control since antagonize deleterious microorganisms [12, 30].

Efficacy of the different nutrient solution amendments (i.e. – Neem, Truban and N-Serve) against rot root caused by *P. capsici*, ranged from effective with Truban and N-Serve treatments in both repeated experiments, to marginally effective with Neem oil in the second experiment, to ineffective with Neem oil in the first experiment. The lack of efficacy of Neem oil amendment in the first experiment can be attributed to the inability of the treatment (both active ingredient and fluorescent pseudomonad increase) to stop the spread of zoospore propagules in the nutrient solution as proved by the results from the baiting essays (Table 3.2). On the contrary, N-Serve and Truban consistently decrease the zoospores presence, thus their spread in the recirculating nutrient solution, which would explain the better disease control obtained in the present and in our previous studies [15].

It is interesting that, the Neem oil amendment had the highest pseudomonad population (*P. putida* and *P. maculicola*) increase, but had the lowest siderophore

production (Fig. 3.5) with fluorescence, similar to the pseudomonad population recovered from the control treatment and that this incidents concurred and resulted in the poorer disease control. This is in agreement with our previous investigation [15] in which we hypothesized the importance of metabolites such pyoverdines in the disease control especially in the hydroponic system. Thus, it would be interesting to investigate why different chemical amendments, while stimulated the same population size of indigenous fluorescent pseudomonads, nevertheless produced isolates with different fluorescence intensity (i.e. siderophores production) on the same KB substrate. Siderophores have a role in the antagonistic activity of several pseudomonads [12, 30]. Besides this, the production of other metabolites with an antagonistic action may also have been produced when the population size of the pseudomonads increased to such extent (2-3 log CFU ml^{-1}) in the nutrient solution. Signal molecules, such as N-acyl homoserine lactones (AHL), produced when the bacterial population reaches high densities, has been associated to the antibiotic production [7].

In conclusion, we have shown that synthetic and natural nitrification inhibitors were consistent, in selectively enhance fluorescent pseudomonad population in the nutrient solution and rhizoplane, so to reduce pathogen population (Table 3.2) and increase plant survival (Fig. 3.7). Use of this strategy has the potential to overcome inconsistencies due to lack of survival and colonization seen in previous studies. These formulations however need to be investigated more deeply to identify the right concentration of active and inert ingredient to be combined to obtain the best result. In our study, addition of 50 $\mu l\ l^{-1}$of the commercial product Neem KillerB to the nutrient solution (after *in vitro* test at different concentration; data not showed), was found to be the optimal amount for the bacterial increase (2-3 log units) also in the greenhouse. However, such amount contains only 0.075 $\mu g\ l^{-1}$ of the active ingredient, thus too low to play a significant part in the disease control. The different effect of the active vs. inert ingredient contained in their formulation and their synergistic effect on the pathogen as well as to the indigenous microflora need to be addressed together with the mode of action that the fluorescent pseudomonad can have as biocontrol.

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Table 3.1. Summary of treatments and replications in each of two experiments to assess the efficacy of different treatment on *Phytophthora capsici* in pepper plants in recirculating cultural systems

Treatment	Concentration (µg ml^{-1})	Replications [a] per experiment	
		Experiment 1	experiment 2
Control		2	2
Phytophthora [b]		3	3
Phytophthora + N-Serve24[c]	12.5	3	3
Phytophthora + Neem[c]	0.075	3	3
Phytophthora + Truban [c]	12.5	3	3
Phytophthora + N-Serve24[c] + Pf5 [d]	12.5	3	3

[a] Each replication consisted of drip-irrigated system (unit) with 2 troughs connected to a 25-liter reservoir; each trough contained 4 pepper plants potted in organic potting mix.
[b] *Phytophthora capsici* were introduced into the hydroponic system between the second and the third chemical application by hypocotyl inoculation of one plant in the system.
[c] N-Serve®, Truban® and Neem oil treatments were added directly to the nutrient solution.
[d] Biocontrol Pf5 at 4-4.5 log CFU ml^{-1} was added to the N-Serve® treatment just after the chemical amendment.

Figure 3.1. Drip irrigated hydroponic units in a temperature-controlled greenhouse (A) and mortality of pepper plants after hypocotyls-inoculation of a single plant on one side of a two-sided recirculating hydroponic units in the absence (right) or presence of a nitrification inhibitor (left) (B).

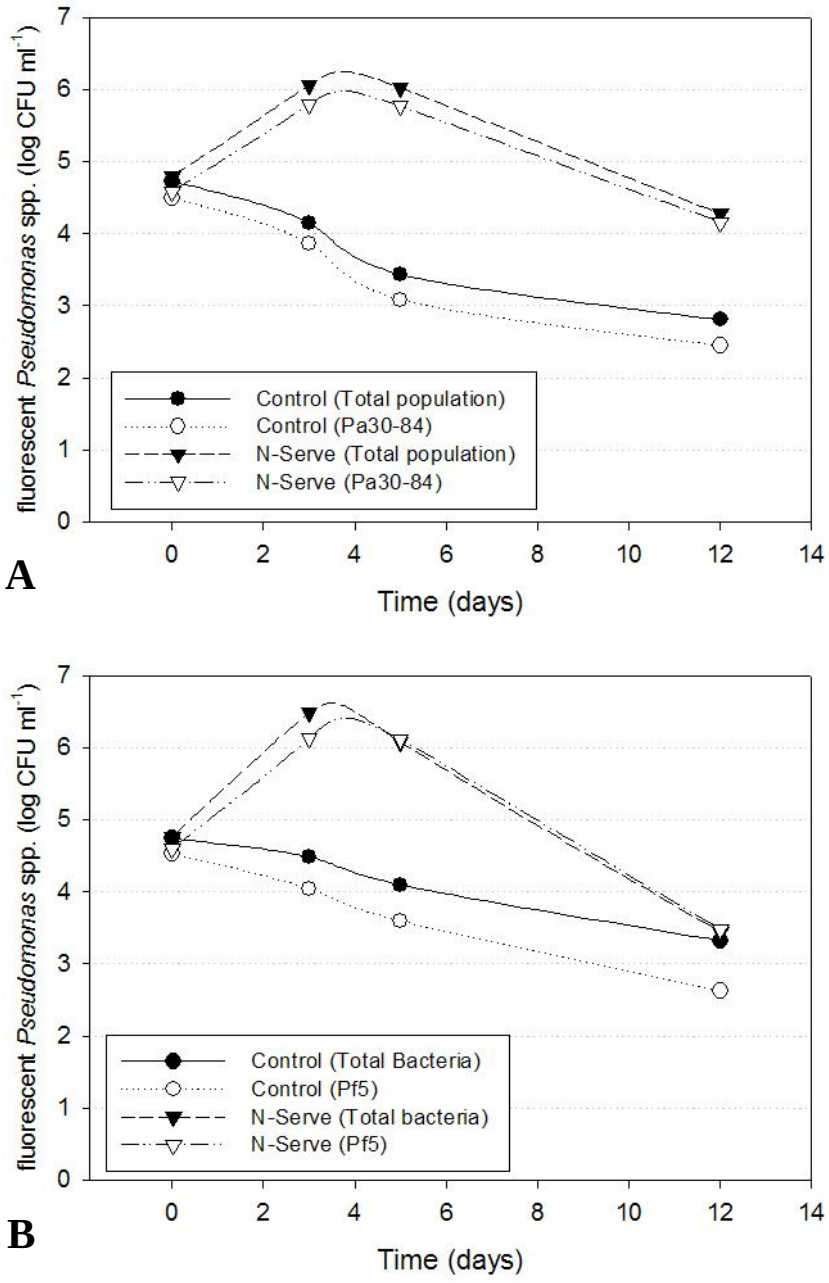

Figure 3.2. *In vitro* effect of N-Serve® on the population of biocontrols isolates *Pseudomonas aureofaciens* Pa 30-84 (A) and *Pseudomonas fluorescent* Pf5 (B) in nutrient solution in presence of indigenous fluorescent pseudomonads. Hydroponic nutrient solution samples from pepper greenhouse experiment were treated *in vitro* and compared with controls. 4.5 log CFU ml^{-1} of Pf5 Rif+ or Pa 30-84 Rif+ were added to the nutrient solution with or without N-Serve amendment at 12 µg a.i. ml^{-1}.

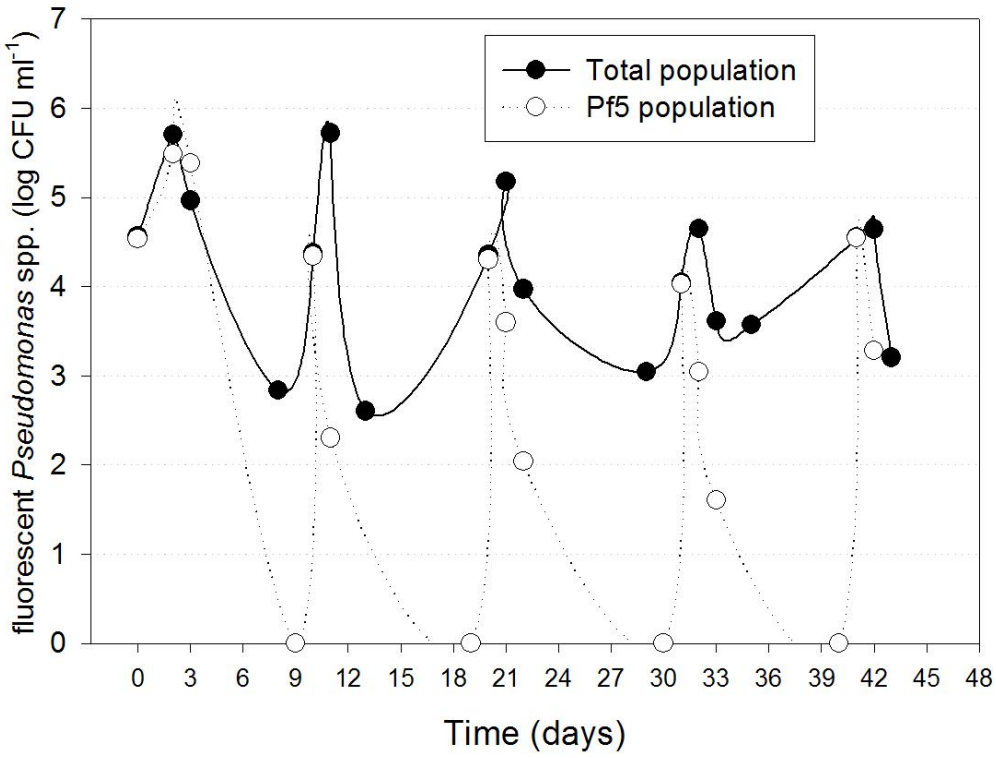

Figure 3.3. *In vivo* effects of N-Serve® on the biocontrol isolate *Pseudomonas fluorescent* Pf5 in recirculating nutrient solution in the presence of indigenous fluorescent pseudomonads. Nutrient solution was treated with N-Serve® (12.5 µg a.i. ml^{-1}) and amended with the biocontrol *Pseudomonas fluorescent* Pf5 Rif+ at 4-4.5 log CFU ml^{-1}.

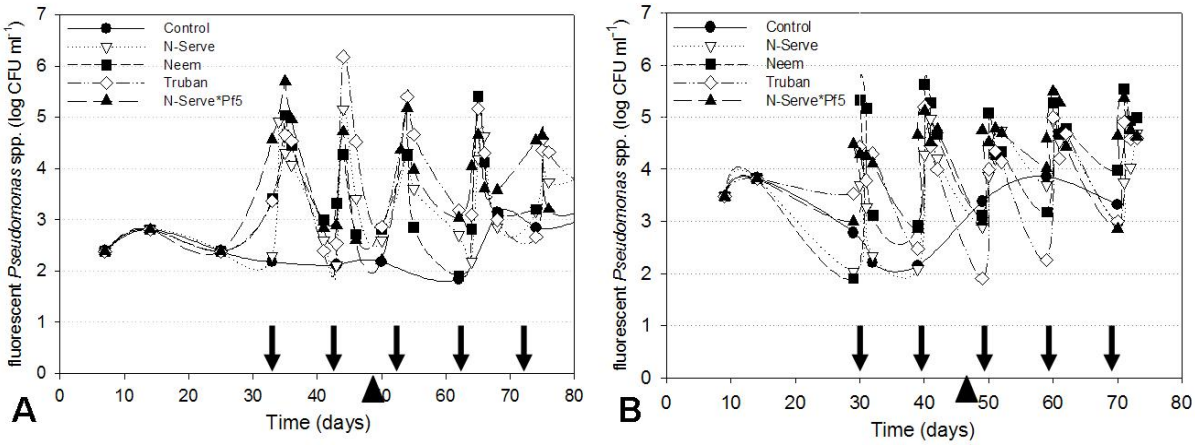

Figure 3.4. Mean population densities of fluorescent Pseudomonads in a drip irrigation hydroponic system with pepper as the host plant in the 1st (A) and 2nd (B) experiment. Down arrows indicate the timing of treatment applications. Up head arrow indicate *P. capsici* inoculation. Treatments consisted of nutrient solution not amended or amended with N-Serve, Neem, Truban and N-Serve plus Pf5 isolate at 4-4.5 log CFU ml^{-1}.

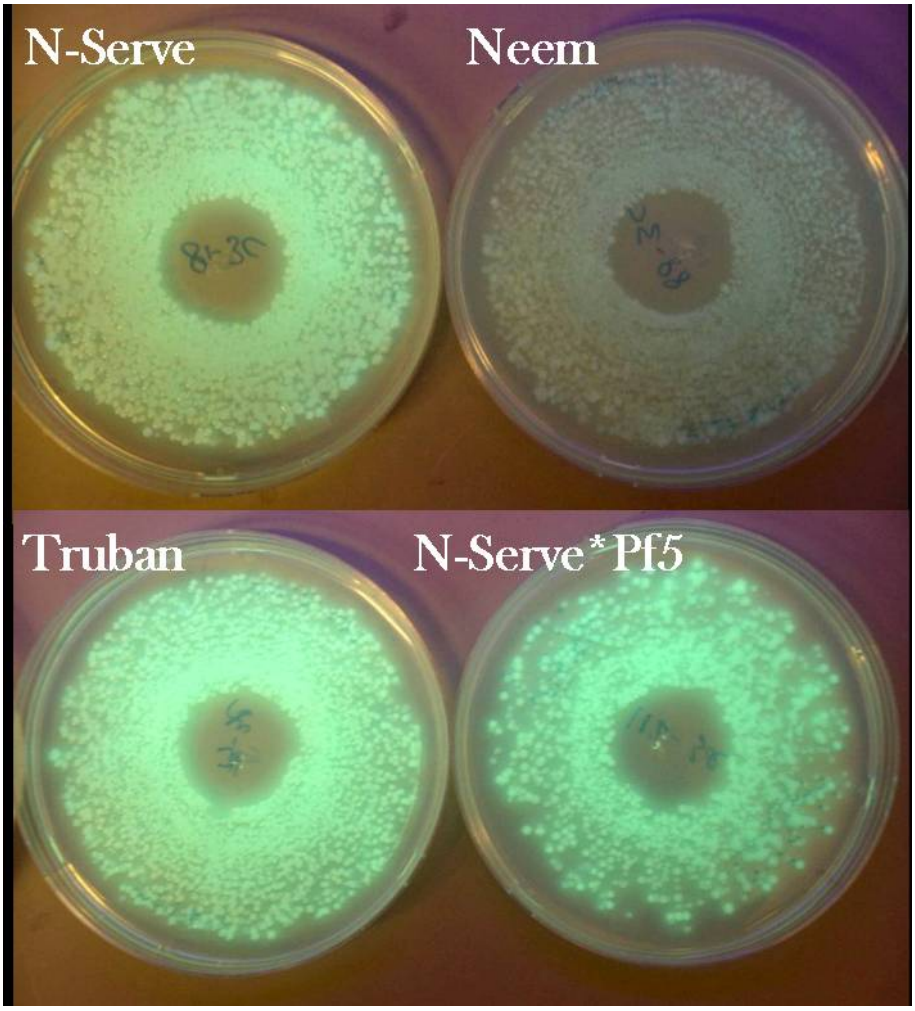

Figure 3.5. Fluorescence produced on KB medium under UV light at 600 nm (which indicates siderophores production), by pseudomonads recovered from nutrient solution amended with different nitrification inhibitors.

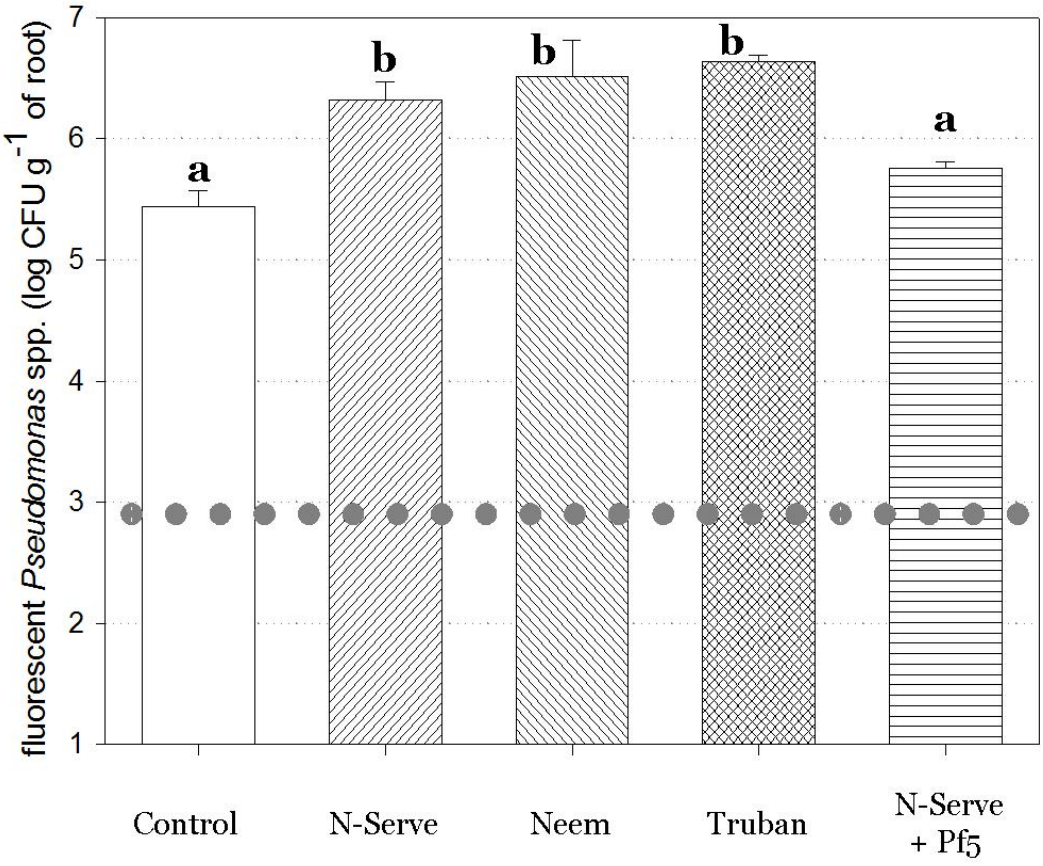

Figure 3.6. Population densities of fluorescent pseudomonads recovered from root of pepper plants growth in a drip irrigation system, 10 days after the last application of the treatments. Treatments consisted of nutrient solution not amended or amended five times with N-Serve® at 12.5 µg a.i. ml^{-1}, Truban at 12.5 µg a.i. ml^{-1}, Neem (50 µl of commercial product) and N-Serve® plus *P. fluorescent* Pf5 Rif+ at 4-4.5 log CFU ml^{-1}. Bars with different letters are significantly different at P=0.05 according to the All Pairwise Multiple Comparison Procedures (Student-Newman-Keuls method). Error bars represent the standard error of the mean (n = 4). Horizontal dots indicate population densities of the fluorescent pseudomonads in the nutrient solution at the roots sampling day.

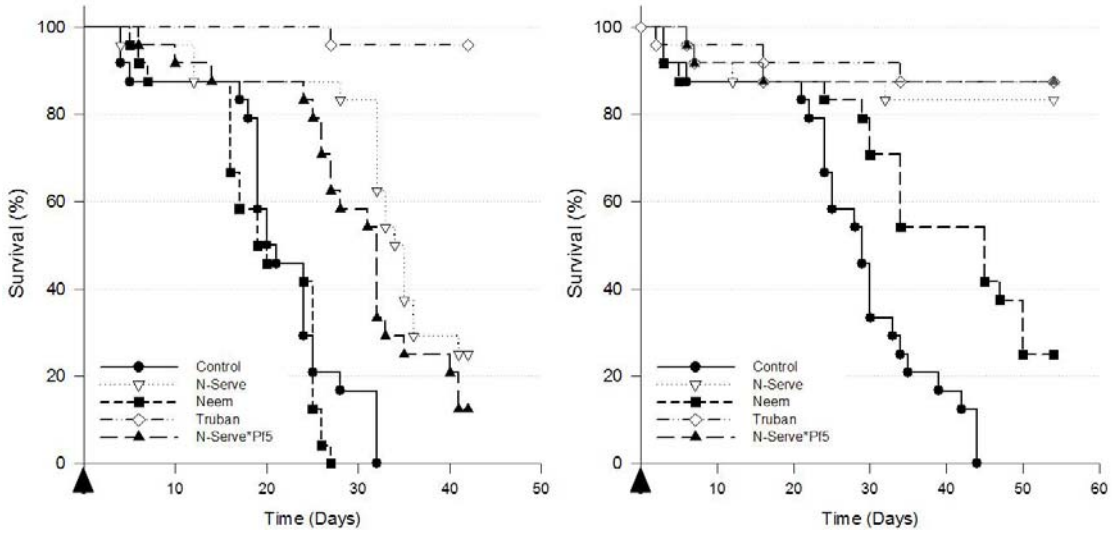

Figure 3.7. Kaplan–Meier estimates of survival functions describing time to death of pepper plants in a drip irrigation hydroponic system after *P. capsici* hypocotyls inoculation. P values were derived with the log-rank test followed by all pairwise multiple comparison procedure (Holm-Sidak method) to isolate groups that differ from each other. Treatments consisted of nutrient solution not amended or amended five times with N-Serve, Neem, Truban and N-Serve plus Pf5 isolate at 4-4.5 log CFU ml-1. Blockade treatment was applied as a foliar spray application.

Table 3.2. Baiting assay for the detection of zoospores in the recirculating nutrient solution from pepper hydroponic systems after *Phytophthora capsici* hypocotyls inoculation. Treatments consisted of nutrient solution not amended or amended with N-Serve, Neem, Truban and N-Serve plus Pf5 isolate at 4-4.5 log CFU ml^{-1}.

	Baiting assays (% of colonized leaves)		
Treatment	1st (10 days)	2nd (20 days)	3rd (30 days)
	First experiment		
Control	-	0[a] nt	0 nt
Phytophthora	-	68 a	87 a
Phytophthora + N-Serve	-	18 b	10 b
Phytophthora + Neem	-	78 a	100 a
Phytophthora + Truban	-	0 b	0 b
Phytophthora + N-Serve + Pf5	-	23 a b	52 a
	Second experiment		
Control	0 nt	0 nt	0 nt
Phytophthora	12 a	17 a	25 a
Phytophthora + N-Serve	2 a	13 a	2 a
Phytophthora + Neem	7 a	27 a	7 a
Phytophthora + Truban	0 a	0 a	0 a
Phytophthora + N-Serve[c] + Pf5	2 a	2 a	7 a

[a] Multiple comparisons of means (n=3) within a column in the same experiment are based on the One Way Analysis of Variance or Kruskal-Wallis One Way Analysis of Variance on rank tests. Values followed by the same letter are not significantly different at P = 0.05. nt, not tested.

GENERAL CONCLUSION TO THESIS DISSERTATION

The overall objective of this thesis was to evaluate alternatives integrated disease management (IDM) strategies for zoosporic pathogens in recirculating systems. The results from this study demonstrates the possibility of using a specific substrate to selectively enhance and maintain desired populations of natural-occurring bacterium such *Pseudomonas* spp., a trait considered to have great potential in biocontrol applications for plant protection. Our findings indicated that synthetics and naturals nitrification inhibitors, containing active and inert ingredients, could serve (directly or indirectly) as biological and chemical control at the same time, since they exhibited bi-functional properties.

Briefly, the main findings of this thesis can be summarized as follows:

Within 48-72 hrs after amending the nutrient solution with either N-Serve, Truban or Neem oil, irrespective of concentrations, the population of fluorescent pseudomonads increased from 10^3 to 10^5-10^6, with a peak population ca. 3 days after application. The population slowly declined over the next 10 days until the chemical was reapplied and the pattern was repeated. This response occurred in each of thirty experiments involving gerbera, pepper, cucumber, (Arabidopsis, coleus, chrysanthemum; data not showed) in different greenhouses and at different times of the year. Conclusion: The phenomenon is repeatable and predictable;

We demonstrated that nitrapyrin, the active ingredient in N-Serve®, exerted direct antifungal activity whereas inert ingredients had an indirect role in disease suppression since were responsible for the increase in the fluorescent pseudomonad population in the nutrient solution but also in the plant rhizoplane. Specifically, although both the active ingredients (nitrapyrin) and the inert ingredients (xylene and 1,2,4-Trimethylbenzene; i.e., the inert ingredients in N-Serve®) can serve as the substrates associated with population increases of the bacterium, xylene and 1,2,4-trimethylbenzene were responsible of a significant percentage of the fluorescent pseudomonad population increase while nitrapyrin cause only a slight enhancement. However, those increases were significantly higher when the active ingredient (i.e., nitrapyrin) and the inert ingredients were combined, which suggests a synergistic response.

The resident fluorescent *Pseudomonas* spp. enhanced by the amendment was identified, via conventional as well as molecular techniques, as *Pseudomonas putida.* Amending the recirculating nutrient solution with a representative fluorescent pseudomonad isolate verified and substantiated their role in disease control. This result indicates that foreign biocontrol agents do not need to be added. They already exist in most habitat, thus we only need to selectively enhance them.

The resident indigenous fluorescent *Pseudomonas* spp., were more competitive (as estimated by maximum population levels attained), than two known biocontrol isolates of *Pseudomonas* spp. (Pa 30-84 and Pf5) when they were seeded into the nutrient solution together with the chemical amendment.

Both N-Serve and Truban amendment to the nutrient solution provided disease control of both *Phytophthora capsici* on pepper, and *Pythium aphanidermatum* on cucumbers in replicated and repeated experiments over a tree year period. 100% control was achieved in the ebb-and-flow systems; 100% control or significant delay of the disease was realized in drip irrigated cultural and in floating systems. In the absence of the amendments, all plants died.

Furthermore, amendment of the recycled nutrient solution with nitrification inhibitors (N-Serve and Truban) had a stabilizing effect on the pH and electrical conductivity and promising positive effects on plant growth and yield (Appendix A).

In conclusion, we have shown that synthetic and natural nitrification inhibitors were consistent, in selectively enhance fluorescent pseudomonad population in the nutrient solution and rhizoplane, so to reduce pathogen population and increase plant survival. Use of this strategy has the potential to overcome inconsistencies due to lack of survival and colonization seen in previous studies. These formulations however need to be investigated more deeply to identify the right concentration of active and inert ingredient to be combined to obtain the best result.

APPENDIX A

Improving Crop Quality and Minimizing Nitrate Leaching Through Manipulation of Ammonium Nitrate Ratios of Fertilization Programs

Deborah Pagliaccia, Donald Merhaut

(Part of the final report submitted to the KKRF in 2005)

Research projects funded by the Kee Kitayama Research Foundation (KKRF)
http://www.corf.org/research.html

GENERAL INTRODUCTION

The primary goal of the present research is to mitigate nutrient runoff from nursery production sites. To reach this goal, the laboratory studies encompass: (1) the determination of nutrient uptake dynamics during crop production so that fertilizer can be applied at the rates needed during the production periods when crops are actively taking up nutrients; (2) the development and refinement of 'closed' production systems such as hydroponic culture, where nutrient solutions are completely recycled; (3) the development of production methods which utilize more ammoniacal- nitrogen (NH_4-N), which does not leach as readily as nitrate-nitrogen (NO_3-N); and (4) the development of products that can be used in ornamental production which minimize nitrification ($NH_4 \rightarrow NO_3$).

One of the initial objective was to look at the effects of nitrogen form (NH_4, NO_3) on crop development in lilies, lisianthus and limonium, with the goal of possibility utilizing more NH_4-N than NO_3-N (research programs 2 and 3). The first attempt to grow lilies hydroponically in winter 2002-2003 was not successful. The primary failure was that the bulbs were submerged in nutrient solution. These bulbs rotted even though the nutrient solutions were well aerated. The study was repeated in winter 2004, with the same closed recirculating system, but using drip irrigation with bulbs in perlite rather than being submerged in solutions. Research with Lisianthus and limonium will resume in the spring after the lily crop.

During the fall of 2003, a visiting scientist, Deborah Pagliaccia, joined Dr. Merhaut laboratory and started to conduct studies (fall 2003, spring 2004) on the growth of

floriculture crops, through funding provided by her government. With the hydroponic system designed and constructed from KKRF funding, she has investigated the use of the nitrification inhibitor, N-Serve, in hydroponically grown gerberas. Gerbera was selected as the model plant since this is a major crop in the United States and her country. The research report of that study is presented below:

The Effect of N-Serve, a Nitrification Inhibitor, on Gerbera Growth, Flower Production, nutrient solution pH and Electrical Conductivity (EC) in Hydroponic Production Systems

INTRODUCTION

Greenhouse growers are facing strict governmental regulations concerning the discharge of spent nutrient solution in order to abate ground water pollution resulting from nutrient salts and agricultural chemicals. At the same time the use of recaptured irrigation water by the agricultural/horticultural industry is increasing due to increased pressure by water purveyors and governmental agencies to reduce water usage. When used as a completely closed recirculating system, the above would seem to be a solution to the problem of pollution and water conservation. Although the use of recycled irrigation water will reduce water usage and ground water pollution, serious grower concerns exist regarding the spread of phytopathogenic and deleterious microorganisms in the recycled water.

The first objective of the present study was to evaluate the *in vitro* effect of N-Serve, a nitrification inhibitor (see Chapter 1 for details), on zoospores motility and viability of *Phytophthora cryptogea.* Second, we evaluated the *in vivo* effects of N-Serve on the culturable aerobic heterotrophic bacteria (AHB) and its effect on pH and electrical conductivity (EC) on the hydroponic nutrient solution used in the cultivation of gerbera. Additionally, N-Serve effects on plant growth and yield of gerbera was evaluated.

MATERIALS AND METHODS

Laboratory studies.

The effect of N-Serve on **zoospores motility of *Phytophthora cryptogea*** was determined by comparing the duration of motility in nutrient solution amended with N-Serve with that of a control preparation. One ml of zoospore suspension with approximately 1000 zoospores was placed in triplicate in the 60 mm diameter Petri dishes. N-Serve at 0; 5; 10; 25; 50 and 100 µg a.i. ml^{-1} concentration was then applied to the nutrient solution observing simultaneously under the microscope the motility length of the zoospores at 6.6X (Zeiss Stemi SV11 Apo). Zoospores germination (viability) in solution was observed after 24 hours.

The efficacy of N-Serve on the **zoospores germination of *Phytophthora cryptogea*** was tested on substrate 20%V8 juice agar (VJA). The chemical were added to autoclaved liquid V8JA when the agar had cooled to approximately 45 C°. A 200 µl suspension of zoospores cysts, with approximately 150 zoospores were plated onto the surface of Petri dishes with V8JA amended with N-Serve at 0, 1, 10, 100, 0.3, 3 and 30 µg a.i. ml^{-1}. Un-amended V8JA was used as a control. Cultures of *Phytophthora cryptogea* were incubated at room temperature and after 48h, the number of colonies (i.e. germinated zoospores) appearing on each plate at each concentration were recorded observing the plates under a microscope (Zeiss Stemi SV11 Apo) at 40X. There were three replicate plates per treatment and the experiment was repeated twice. The active ingredient nitrapyrin is sparingly insoluble in water, so it was dissolved in organic solvent. During this study dimethyl sulfoxide (DMSO) was utilised as solvent.

Greenhouse Studies

Hydroponic studies. Experiments were conducted in closed recirculating hydroponic cultural systems in temperature-controlled greenhouses (20-32°C). Gerbera plants (*Gerbera jasemonii* cv. Timo) was used as the host plant. There were two gerbera experiments and each consisted of two treatments: N-Serve®-amended and non-amended nutrient solution at 25 µg a.i. ml^{-1}. All other details on the cultural system employed for these experiments are described in chapter 1, including nutrient solution components and maintenance, growth of gerbera plants. During the course of the experiments period, plants

were monitored for flower production and nutrient solutions were monitored for pH, electrical conductivity. At the end of the experiments all plants were harvested, shoots were separated from roots and both were oven-dried at 70C (158F) to record dry mass.

Additionally, we monitored the effect of N-Serve on the AHB populations in amended and unamended systems. Samples of the nutrient solution were collected at different interval and the bacteria populations were estimated by plating a dilution series onto Tryptic soy agar (see Chapter 1 for details).

RESULTS

Laboratory studies: Preliminary *in vitro* tests indicated that the minimal N-Serve concentration required to stop zoospore motility of *P. crytogea* in nutrient solution, is approximately 10 µg a.i. ml^{-1}. Above 10 µg a.i. ml^{-1} all concentrations tested, affected zoospore motility (Table A.1) with a total motility cessation within 30 second after being placed in solution containing N-Serve. Zoospores not treated or treated at 5 µg a.i. ml^{-1} with the chemical retained their motility.

Germination on V8JA substrate of motile zoospores of *P. crytogea* was significantly reduced with 30 and 100 µg a.i. ml^{-1} N-Serve concentrations compare to the treated agar with 0, 1, 0.3, 3, and 10 µg a.i. ml^{-1} (Fig. A.1). N-Serve at 30 µg a.i. ml^{-1} reduced zoospore germination by 50% compared to 0 µg a.i. ml^{-1}. 90% reduction of zoospores germination was obtained with 100 µg a.i. ml^{-1}.

Greenhouse Studies

Monitoring bacterial populations in the nutrient solutions. Significant increases in the general bacteria population (AHB), compared to the non-amended control treatment, were consistently recorded in both experiments following the addition of N-Serve® to the recirculating nutrient solution (data presented in Chapter 1). Additionally, our preliminary study indicated that the bacterial species diversity also changed following application of N-

Serve. Specifically there was a significant increase of fluorescent pseudomonads in the N-Serve treatment compare to the control (data not showed).

Nutrient Solutions pH and EC. The pH of nutrient solutions without N-Serve decreased during the studies (Fig. A.2). Therefore, the pH of these solutions had to be corrected to pH 6.0 with the addition of potassium hydroxide. Nutrient solutions supplemented with N-Serve were stable throughout the period of the study (Fig. A.2.). The electrical conductivity of nutrient solutions gradually increased from 1.6 mS/cm to 1.8 to 2.3, depending on the treatment (Fig. A.3). However, the EC of nutrient solutions without N-Serve showed to increase more compare to the N-Serve treated solution during the study (Fig. A.3).

Plant growth and yield. Plants treated with N-Serve had significantly larger shoots dry weight (P=0.001) compare to the control (Fig. A.4 - data from 2 repeated experiments were combined). In the 1st experiment, root dry weights and flowers production (Fig. A.5 and fig A.6 - data from 2 repeated experiments were not combined) was significantly increased (P= 0.0009 and P=0.002 respectively) in N-Serve amended system compare to the control while no difference was observed between treatments in the 2nd experiment (P= 0.068 and P=0.75 respectively).

Note. Based on the above results, additional investigations were conducted in Dr. Stanghellini's laboratory (2004-2005) to elucidate the efficacy of N-Serve as a fungicide and as a carbon source for the bacterial populations in the recycled nutrient solution, and its effect on plant growth and yield.

Further experiments conducted in drip recirculating systems using Gerbera plants (*Gerbera jasemonii*) and Truban (See Chapter 2 for details) amendment to the nutrient solution corroborated the stabilizing effect on the pH, electrical conductivity and the positive effects on plant growth and yield following the use of nitrification inhibitors.

N-Serve (µg ml^{-1})	Stop motility	Germination after 24 h
0	No	Yes
5	No	Yes
10	Yes	Yes
25	Yes	Yes
50	Yes	Yes
100	Yes	Yes
DMSO	No	Yes

Table A.1. Motility and visual germination of zoospores of *Phytophthora cryptogea* in nutrient solution amended or not amended with N-Serve.

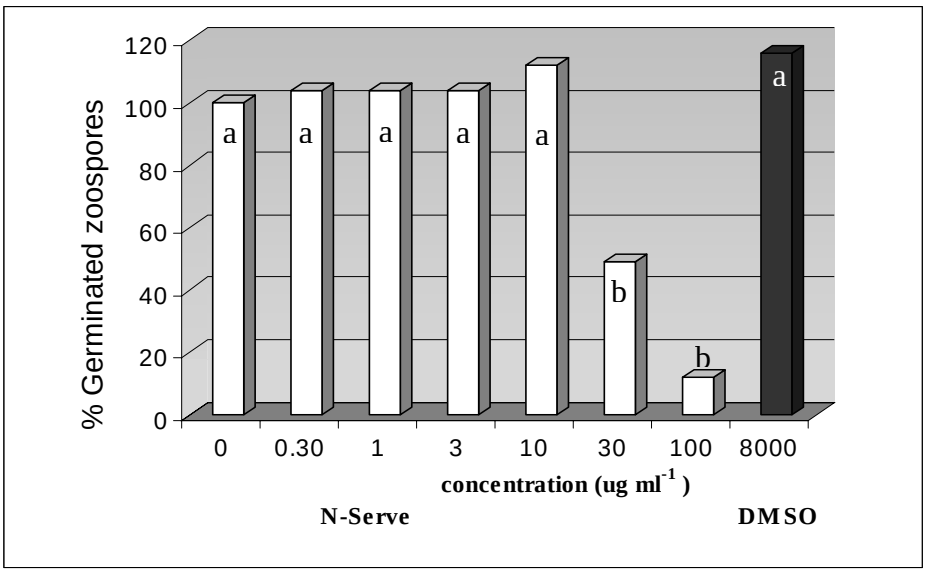

Fig. A.1. Direct germination of zoospore cyst of *Phytophthora cryptogea* on V8JA substrate in the presence of N-Serve. Un-amended V8JA was used as a control. Values are the means of three replicates for two experiments. Bars with different letters are significantly different at P = 0.01 according to the All Pairwise Multiple Comparison Procedures (Holm-Sidak method).

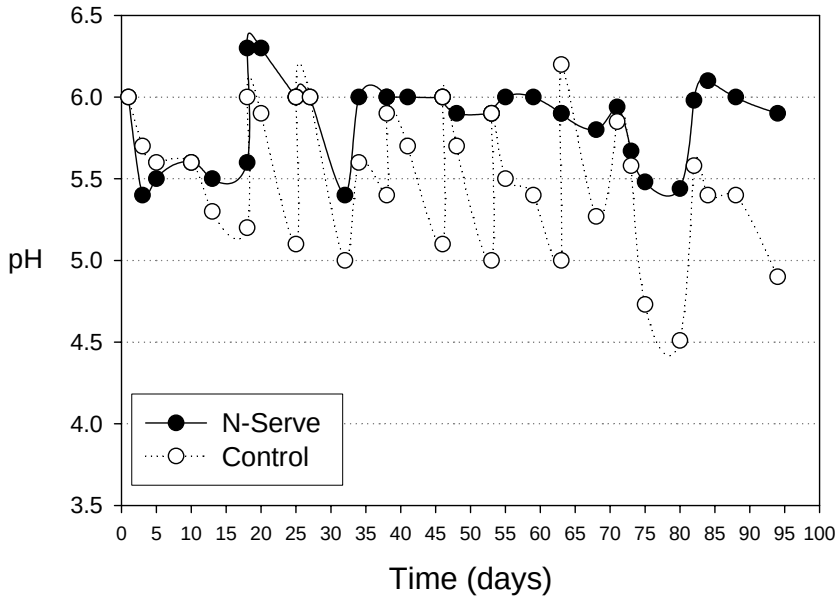

Fig. A.2. Solution pH as affected by N-Serve versus the control during a three month production cycle. Potassium hydroxide was added to nutrient solutions when pH dropped below 5.5. Two experiments were performed; the figure shows one representative experiment.

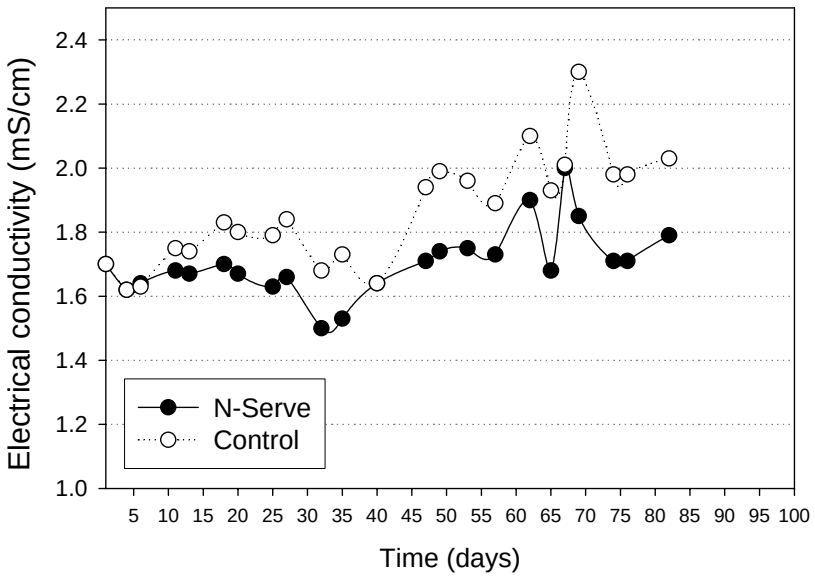

Fig. A.3. Solution EC as affected by N-Serve versus the control during a three month production cycle. Water was added to nutrient solutions when EC rise above 1.8-2.0 (mS/cm). Two experiments were performed; the figure shows one representative experiment.

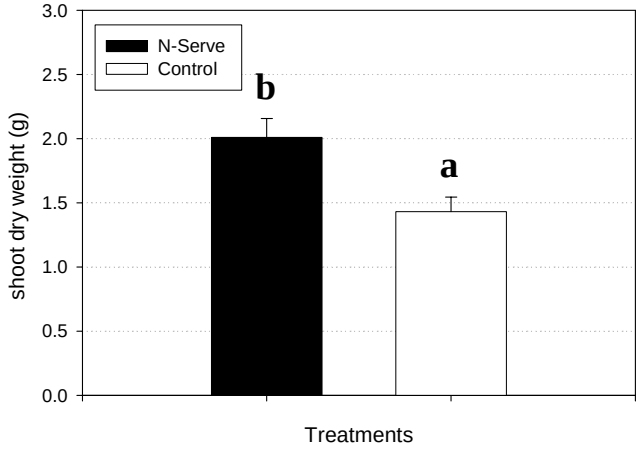

Fig. A.4. Shoot dry weight (g) of gerbera plant (cv Timo) grown on treated (N-Serve) or untreated (Control) recycled nutrient solution. Bars with different letters are significantly different at P = 0.05 according to the Paired t-test. Data represent mean ± SE (n = 36). The figure shows means data from two repeated experiments.

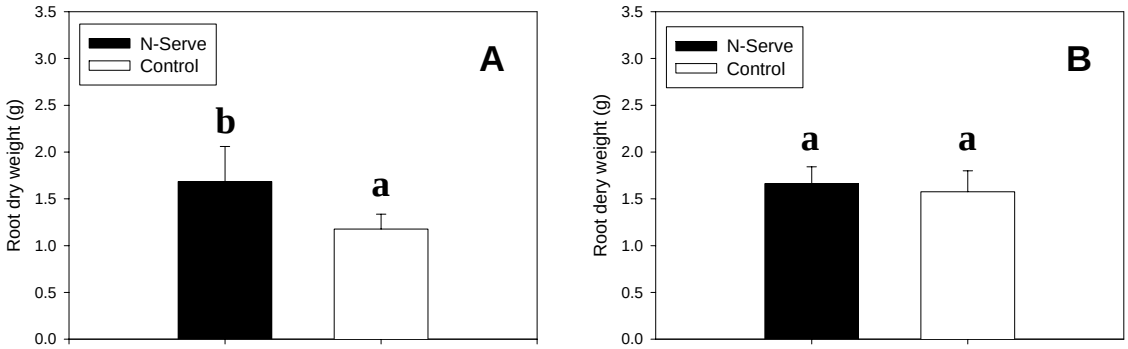

Fig. A.5. Root dry weight (g) of gerbera plant (cv Timo) grown on treated (N-Serve) or untreated (Control) recycled nutrient solution. Bars with different letters are significantly different at P = 0.05 according to the Paired t-test. Data represent mean ± SE (n = 18).

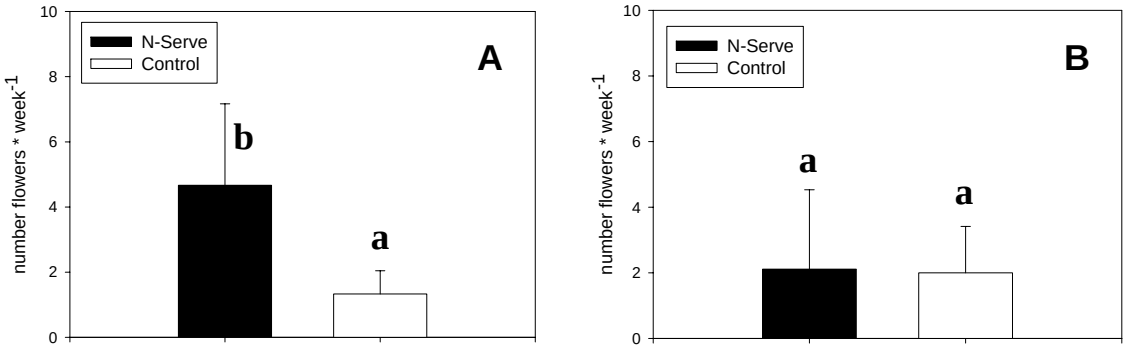

Fig. A.5. Flower production of gerbera plant (cv Timo) grown on treated (N-Serve) or untreated (Control) recycled nutrient solution. Bars with different letters are significantly different at P = 0.05 according to the Paired t-test. Flowers were harvested once per week. Data represent mean ± SE (n = 9).

APPENDIX B

OBJECTIVE

1. To determine *in vitro* effect of N-Serve and Terrazole (etridiazole a.i., for details see Chapter 2), at 100 µg a.i. ml^{-1} concentration, on nitrifying bacteria (Nitrosomonas) in *Nitrosomonas europaea* (*Ne*) media;

2. To determine *in vitro* effect of N-Serve and Terrazole, at 50 µg a.i. ml^{-1} concentration, on nitrifying bacteria (Nitrosomonas) in hydroponic nutrient solution;

3. To determine presence or absence of ammonia (AOB) oxidisers bacteria population in gerbera hydroponic nutrient solution from commercial nursery (Dramm & Echter, Inc., Encinitas; California).

METHODS

1. A 1% inoculum of a 3 day old *Nitrosomonas europaea* culture was used to inoculate *Nitrosomonas europaea* (*Ne*) media at a pH of 7.5 that contained either no fungicide, 100 µg a.i. ml^{-1} Terrazole or 100 µg a.i. ml^{-1} N-Serve. Cells were incubated at 30 C for 3 days on a rotary shaker (200 rpm) and sampled twice daily for nitrite. After the third day, 400 ml of media containing Terrazole, N-Serve plus cells and 200 ml of media containing just cells were concentrated by centrifugation. The resulting cell pellet was washed, resuspended and used to inoculate fresh medium without fungicide. Nitrite accumulation was monitored for the next 3 days to gage whether nitrification activity could be recovered from the fungicide treatments.

2. A 1% inoculum of a 3 day old *Nitrosomonas europaea* culture was used to inoculate a hydroponic nutrient solution at a pH of 6 that contained either no fungicide, 50 µg a.i. ml^{-1} Terrazole or 50 µg a.i. ml^{-1} N-Serve. Cells were incubated at 30 C for 3 days on a rotary shaker (200 rpm) and sampled once daily for nitrite. After the third day, 400 ml of solution containing Terrazole, N-Serve plus cells and just cells were concentrated by centrifugation. The resulting cell pellet was washed, resuspended and used to inoculate fresh *Ne* medium without fungicide. Nitrite accumulation was monitored for the next 4

days to gage whether nitrification activity could be recovered from the fungicide treatments.

3. One liter of recycled nutrient solution from *Gerbera* hydroponic systems, collected at the nursery (Dramm & Echter, Inc., Encinitas; California), was filtered onto 8 and 0.2 um nitrocellulose membranes. Cells were rinsed from the membranes, lysed and the DNA subjected to phenol chloroform extraction and ethanol precipitation. The nucleic acids extracted was then amplified with AOB-specific McCaig PCR (1.1 Kb) and digested with *Alu I.* Restriction digest using DNA from extracted pure cultures of *Nitrosomonas europaea* (*Ne*) and *Nitrosospira multiformis* (*Nm*) were also performed. The digest products were separated on a 2% LMP (low melting point) agarose gel and stained with ethidium bromide.

RESULT AND DISCUSSION

Effect on *Nitrosomonas* growth in *Nitrosomonas* medium (Fig. B.1). When *Nitrosomonas europaea* was inoculated into *Nitrosomonas* medium without chemicals amendment at a 1 % inoculum, nitrite accumulation was observed within the first 16 h and plateau was observed at roughly 14 mM after 3 days of growth. However, nitrification by *N. europaea* was effectively inhibited by the addition of 100 µg a.i. ml^{-1} of either fungicide, Terrazole and N-Serve, to the growth medium. In addition, we were unable to recovery nitrifying activity after these cultures containing either Terrazole or N-Serve were concentrated and resuspended into in fresh media not containing chemicals. This was not surprising because the concentration of 400 mls of media did not result in a reddish pellet as was observed for 200 mls of *N. europaea* alone.

Effect on *Nitrosomonas* growth in hydroponic nutrient solution (Fig. B.2.). None of the treatments, cells alone, Terrazole or N-Serve resulted in nitrification activity when a 1 % inoculum was inoculated into a sterile hydroponic nutrient solution but nitrification activity by *N. europaea* was recovered when 400 mls of the cells alone were concentrated and reinoculated into fresh *N. europaea* medium. As in the previous experiment, we were unable to recovery nitrification activity for either fungicide treatment despite the fact that

the cells were originally incubated with ½ the fungicide concentration of the previous experiment. Combined, these results suggest that both fungicides, N-Serve and Terrazole, permanently render cells inactive, perhaps even killing them.

Presumably the pH of the hydroponic solution (pH 6) was too acidic to support nitrification by *N. europaea*. A significant lag period was observed for growth when cells were reinoculated into the fresh *N. europaea* medium, - i.e. It took three extra days for nitrite to accumulate to 14 mM compared to 1 as in the previous experiment. These data suggest that there was either a lag in gene induction or that the inoculum was less than 1 %. The latter explanation is more likely the case because the concentration of 400 mls of nutrient solution plus cells resulted in a significantly smaller whitish-pellet then that observed for the previous study.

Verification of AOB presence in Gerbera recycled hydroponic solution (Fig. B.3.). The banding pattern generated for restriction digests of AOB-specific DNA amplified from cells filtered onto either 8 or 0.2 um membranes is suggestive of the presence of more *Nitrosospira (Nm)*-like DNA in the Gerbera hydroponic nutrient solution collected from the nursery than *Nitrosomonas (Ne)*-like DNA. The presence of the larger fragment of DNA migrating to ~ 500 bp in the agarose gel differentiates *Nitrosospira* from *Nitrosomonas* DNA in the banding profile. A band of similar molecular weight was observed for the restriction digests of amplified AOB DNAs recovered from the hydroponic solution. However, because the larger fragment migrated to a slightly higher position in the gel than the *Nitrosospira* fragment, it is difficult discern the true identity of the natural AOB population in the Gerbera hydroponic solution without further analysis.

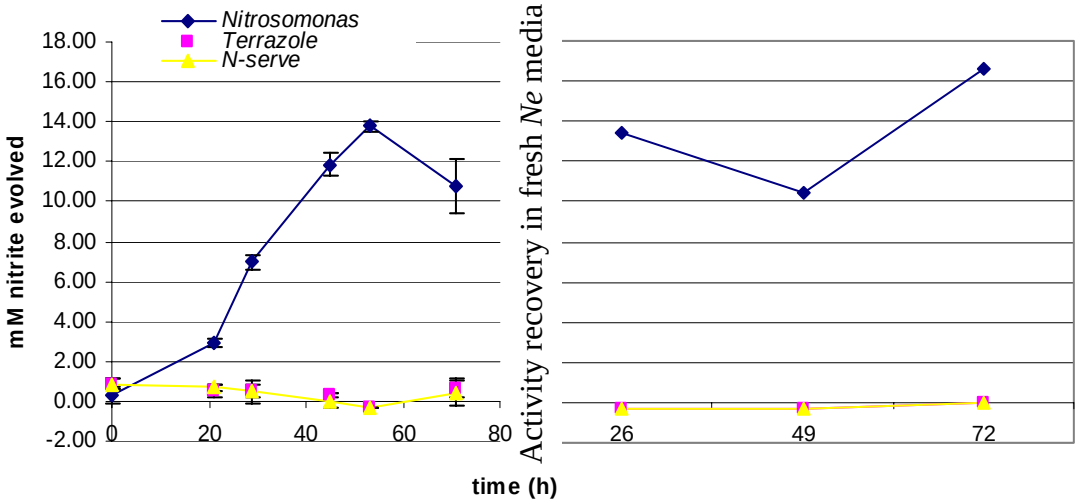

Fig. B.1. Nitrite accumulation following the amendment of *N. europaea* (*Ne*) media with either, no chemicals, 100 µg a.i. ml^{-1} Terrazole or 100 µg a.i. ml^{-1} N-Serve in presence of a 1% inoculum of *Nitrosomonas europaea* culture.

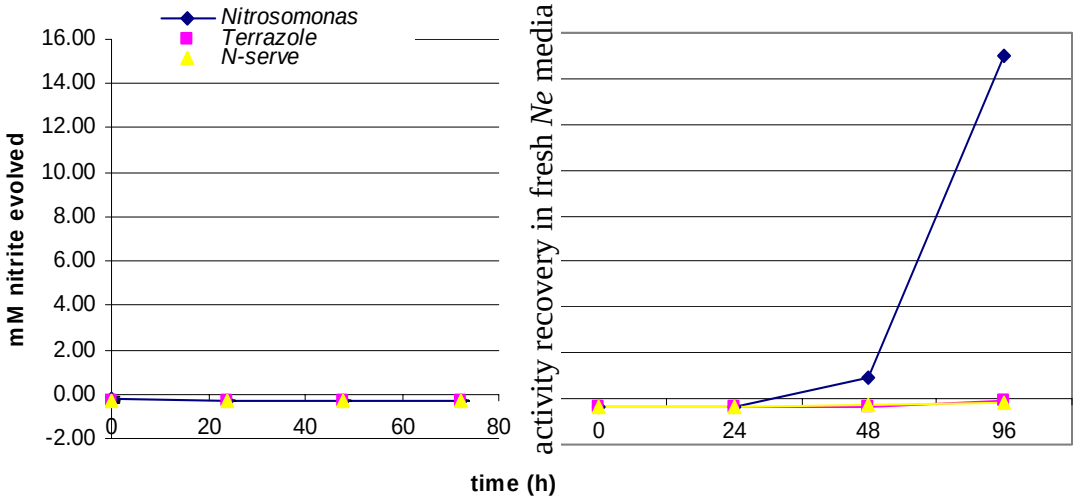

Fig. B.2. Nitrite accumulation following the amendment of hydroponic nutrient solution with either, no chemical, 50 µg a.i. ml^{-1} Terrazole or 50µg a.i. ml^{-1} N-Serve in presence of a 1% inoculum of *Nitrosomonas europaea* culture.

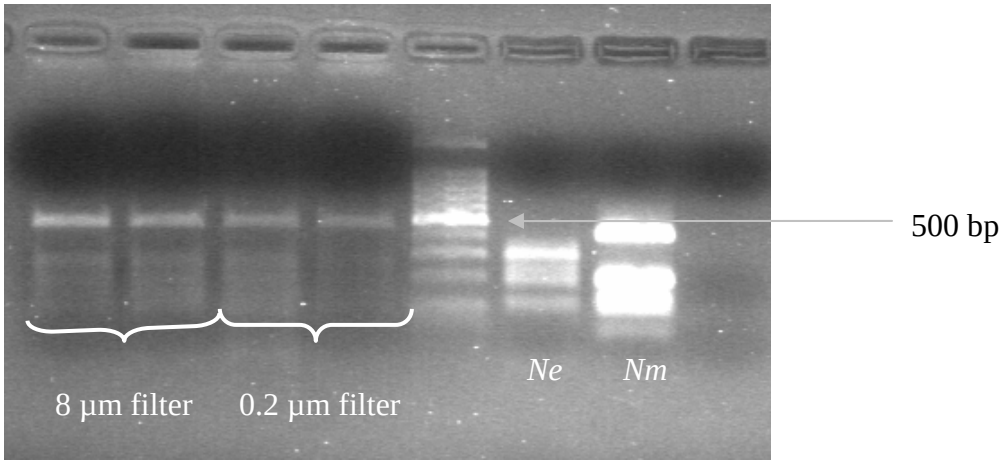

Fig. B.3. *Alu I* restriction digest profiles of AOB-specific McCaig PCR (1.1 Kb) products amplified from nucleic acids extracted from *Gerbera* hydroponic nutrient solution collected in a nursery.