ROLE OF 2,4-DIACETYLPHLOROGLUCINOL-PRODUCING PSEUDOMONAS FLUORESCENS IN THE SUPPRESSION OF TAKE-ALL AND PYTHIUM ROOT ROT OF WHEAT

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To the Faculty of Washington State University:

The members of the Committee appointed to examine the dissertation of RAUL ALLENDE-MOLAR find it satisfactory and recommend that it be accepted.

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Chair

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Abstract

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Take-all decline (TAD) is a field phenomenon in which a spontaneous decline of take-all occurs during continuous wheat or barley monoculture and after an outbreak of the disease. Strains of *Pseudomonas fluorescens* that produce the antibiotic 2,4-diacetylphloroglucinol (DAPG) are not only the major determinant of the suppressiveness of take-all but also in the suppression of other soilborne diseases in many different soils. Soils from fields with a history of crop monoculture, crop rotation or adjacent uncultivated areas were cycled to wheat to "activate" the population of indigenous DAPG-producers. Indigenous populations of DAPG-producing *P. fluorescens* were detected only in soils from fields with a history of wheat, pea or flax monoculture. In soils from fields under crop rotation or non-cropped areas, DAPG producers were below the detection level (log 3.26 CFU/g root) in the rhizosphere of wheat grown in the soils.

To assess the role of DAPG producers in the suppression of take-all, soils that had undergone wheat or pea monoculture, and a non-cropped soil were infested with the take-all pathogen and then the take-all severity on wheat grown in the soils was determined. A greater suppression of take-all was observed in wheat seedlings grown in soils from continuous wheat or pea monoculture fields than in soil from non-cropped fields. Pasteurization of the pea monoculture soil resulted in a loss of DAPG producers and concurrent inability to suppress take-all. Strains of DAPG-producing *P. fluorescens* inhibited *in vitro* mycelial growth of *Pythium* spp.; however, TAD soils from Lind and Quincy, harboring large populations of indigenous DAPG-producing *P. fluorescens,*were not suppressive to Pythium root rot. Pythium root rot severity was similar on "Penawawa" wheat seedlings grown in soils conducive or suppressive to take-all infested with *Pythium* spp. *Pythium* spp. were less sensitive to synthesized DAPG than the take-all pathogen, which may explain the lack of Pythium suppression. In greenhouse experiments, wheat seeds treated with different DAPG-producing *P. fluorescens* genotypes or the recombinant strains Z30-97 or Z34-97, able to produce phenazine-1-carboxylic acid and DAPG, did not reduce Pythium root rot caused by *P. ultimum* or *P. irregulare* in wheat seedlings.

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Dedication

To my wife and my family in Mexico.

GENERAL INTRODUCTION

A suppressive soil is one in which a pathogen, a host and environmental conditions suitable for the development of a disease are present, but if disease occurs it is at a level of incidence and severity lower than expected. The inhospitality of suppressive soils to some plant pathogens is such that "the pathogen does not establish or persist, establishes but causes little or no damage, or establishes and causes disease for a while but thereafter the disease is less important, although the pathogen may persist in the soil" (1). In contrast, conducive (nonsuppressive) soils are soils in which disease readily occurs (34). Suppressive soils occur worldwide and have been described for numerous soilborne plant pathogens including *Fusarium oxysporum* (24), *Gaeumannomyces graminis* var. *tritici* (4, 28), *Pythium* spp. (9), *Streptomyces scabiei* (19), *Rhizoctonia solani* (10, 35), and *Ralstonia solanacearum* (27).

Two different types of disease suppressiveness are known: long standing and induced. Long-standing suppressiveness is naturally associated with the soil, its origin is not known, and it is relatively independent of crop history. In contrast, induced suppressiveness is dependent on agricultural practices such as monoculture (34). Take-all decline (TAD) in wheat is the best known example of induced disease suppression (8). TAD is a field phenomenon that results in a spontaneous reduction in the severity of take-all after an initial outbreak of the disease in fields with continuous monoculture of wheat or barley (34). Although suppressiveness in soil may involve many different organisms, in soils suppressive to take-all in Washington State, USA and The Netherlands, DAPG-producing *P. fluorescens* were found to be responsible for take-all suppression (22, 30).

DAPG-producing strains of *P. fluorescens* also have been reported as responsible for the suppression of other soilborne diseases (13, 20, 25, 26, 31).

Isolates of DAPG-producing *P. fluorescens* within a worldwide collection, have been assigned to 22 distinct genotypes (A to T, PfY and PfZ) based on whole cell repetitive sequence polymerase chain reaction (rep-PCR) with the BOXA1R primer and by restriction length polymorphism (RFLP) and phylogenetic analysis of *phlD*, a key gene in the synthesis of DAPG (7, 11, 15, 16,17, 18). Isolates of DAPG producers differ not only at the molecular level but also in their competitiveness in the rhizosphere. In pea and wheat, the genotype of an isolate is predictive of its ability to establish and maintain threshold rhizosphere population densities (10^5 CFU/g of root), which are required to suppress plant pathogens (7, 12, 13)

The first definitive evidence that an antibiotic produced by *P. fluorescens* is involved in suppression of a plant disease was made by Thomashow and Weller (33). They identified phenazine-1-carboxylic acid as a biocontrol determinant produced by *P. fluorescens* 2-79. This strain, originally isolated from the rhizosphere of wheat, was found to suppress take-all disease caused by the fungal pathogen *Gaeumannomyces graminis* var. *tritici* on wheat. The role of the antibiotic DAPG in disease suppression was first demonstrated in experiments in which a mutant of the DAPG-producing *P. fluorescens* strain F113 defective in DAPG production was unable to protect sugar beet from damping-off caused by *Pythium ultimum* (5).

The host plant influences the population dynamics of bacteria colonizing the rhizosphere (2, 12, 13, 14, 16, 21, 29). For example, in the TAD phenomenon, continuous wheat monoculture leads to a build up of populations of DAPG producers in the

rhizosphere that results in the control of the take-all pathogen (23). Once established, TAD persists as long as monoculture continues (28). The importance of the interaction between the host and beneficial rhizobacteria is further demonstrated by the fact that the TAD suppressiveness can be lost by breaking the wheat monoculture by cultivating oats (28, 34); apparently, a detrimental effect on populations of DAPG producers occurs when oats are grown as observed by the smaller population size of DAPG producers in the rhizosphere of oats when compared to other crops (6). Monoculture of other crops, including pea and flax, also enriches for populations of DAPG producers (11, 13). Landa et al. (11) documented the enrichment of DAPG producers in soil from fields that had undergone more than a century of continuous wheat or flax monoculture but not from fields that had undergone crop rotation. A remarkably observation is that the fields were located side by side and they differed only by the cropping history.

Many *P. fluorescens* strains with biocontrol abilities are introduced onto seeds or planting materials or into the soil to control soilborne diseases. However, once applied into the soil, biocontrol agents usually undergo a reduction in population density due to biotic and abiotic factors including competition, predation, changes in temperature, and availability of nutrients. The reduction of population densities of the introduced biocontrol agent leads to variable colonization and ultimately to inconsistent disease suppression (33). Disease suppression requires the persistence of the introduced biocontrol agent above a threshold density. Furthermore, the amount of disease suppressiveness is directly related to the population density of the biocontrol agent in soil, on roots or in the rhizosphere (3, 22).

Suppressive soils occur worldwide but are underutilized as an approach to control soilborne pathogens (34). Most studies of suppressive soils have focused only on suppression of the target pathogen with little emphasis on effects on other soilborne pathogens. This study focuses on elucidating the breadth of the suppressiveness of TAD and other suppressive soils from the U. S. Pacific Northwest in which DAPG producers occur.

Objectives.

The general objective of this dissertation was to study the role of DAPG-producing *Pseudomonas fluorescens* in the control of take-all and Pythium root rot of wheat.

The specific objective of the first chapter was to determine the population sizes of indigenous DAPG producers in soils that had undergone crop monoculture and to evaluate the effect of these populations on take-all suppression. For this purpose, I cycled wheat in 10 soils samples, including soils from wheat, pea and flax monoculture fields as well as non-cropped areas. I monitored the population dynamics of indigenous DAPG producers in the rhizosphere of wheat. The ability of populations of indigenous DAPG-producing *P. fluorescens* to suppress take-all was evaluated in soils from wheat and pea monoculture fields.

The objective of the second chapter was to determine the role of indigenous DAPG producers from TAD soils in the suppression of Pythium root rot of wheat. TAD soils and virgin soils from two locations in Washington were infested with *Pythium* spp., and then the severity of Pythium root rot was determined.

The objective of the third chapter was to investigate the effectiveness of seed bacterization using DAPG-producing *P. fluorescens* strains of different genotypes, and transgenic *P. fluorescens,* to control Pythium root rot of wheat.

The format for each chapter was based on the requirements of the journal *Phytopathology*.

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Specific Contributions Of The Candidate And His Collaborators

To The Present Studies

CHAPTER 1: Population densities of 2,4-diacetylphloroglucinol (DAPG)-producing *Pseudomonas fluorescens* in monoculture soils: long-term survival and role in take-all suppression.

R. Allende-Molar performed all experiments presented in this chapter. Dr. O. V. Mavrodi, Dr B. B. Landa, and Dr. J. M. Raaijmakers contributed in the planning of the experimental design. Dr. D. Weller contributed in the planning of the experimental design, discussion of results and preparation of the manuscript.

CHAPTER 2: Lack of evidence of control of Pythium root rot of wheat by indigenous 2,4-diacetylphloroglucinol-producing *Pseudomonas fluorescens* from take-all suppressive soils in Washington State.

R. Allende-Molar performed all the experiments presented in this study. Dr. D. Weller and Dr. T. Paulitz contributed in the planning of the experimental design, discussion of results and preparation of the manuscript.

CHAPTER 3: Effectiveness of DAPG-producing *P. fluorescens* genotypes and genetically engineered *P. fluorescens* strains to control Pythium root rot of wheat

R. Allende-Molar performed all experiments presented in this chapter. Dr. T. Paulitz contributed in the planning of the experimental design. Dr. D. Weller contributed in the planning of the experimental design, discussion of results and preparation of the manuscript.

CHAPTER 1

Population Densities of 2,4-Diacetylphloroglucinol (DAPG)-Producing *Pseudomonas fluorescens* in Monoculture Soils: Long-term Survival and Role in Take-all Suppression.

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Keywords: suppressive soils, take-all decline, root colonization, bacterial survival.

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ABSTRACT

Pseudomonas fluorescens strains that produce the antibiotic 2,4-diacetylphloroglucinol (DAPG) are well described biocontrol agents of a wide variety of plant diseases and are responsible for the natural suppressiveness of certain soils. In this study, we quantified the population dynamics and long-term survival of indigenous and introduced isolates of DAPG-producing *P. fluorescens* in soils from non-cropped virgin fields or from fields that had undergone continuous crop monoculture or crop rotation. We also determined the suppressiveness of the soils to take-all. The soils were sown to four successive cycles of wheat each lasting 3 weeks, and population densities of DAPG producers in the rhizosphere were determined at the end of each cycle. The population densities of indigenous DAPG-producing *P. fluorescens* in the rhizosphere of wheat grown in wheat, flax or pea monoculture soils were above the threshold (10^5 CFU/g of root) required for disease suppression. Surprisingly, indigenous DAPG producers remained viable in take-all decline soil from Lind, WA that had been stored for 22 years; population densities of DAPG producers reached the threshold after three cycles. In contrast, DAPG producers were below the level of detection on wheat grown in the virgin and crop rotation soils. Introduced DAPG-producing *P. fluorescens* strains F113 (genotype K) and L5.1-96 (genotype D) survived for one year in a dried soil stored at room temperature and colonized the rhizosphere when wheat was grown. A continuous pea monoculture soil that was known to be Fusarium wilt suppressive, and supported threshold populations of DAPG producers was also suppressive to take-all of wheat. Take-all suppressiveness was transferred by adding the pea monoculture soil into a pasteurized conducive soil (1:9), demonstrating the microbial basis of the

suppressiveness. Pasteurization (60°C 30 min) of the pea monoculture soil resulted in a concomitant loss of DAPG producers and take-all suppressiveness.

INTRODUCTION

Soilborne plant pathogens cause root rots, wilts and damping-off diseases, which are responsible for major losses in food, fiber and ornamental crops throughout the world. These diseases are controlled through the use of fungicides, fumigants, cultural practices such as crop rotation and/or resistant varieties. However, genes encoding resistance to soilborne pathogens are much less common in plant species than resistance genes to foliar pathogens. Furthermore, resistance to some of the most common and widespread soilborne plant pathogens including *Gaeumannomyces* spp., *Pythium* spp. and *Rhizoctonia* spp. are lacking (56).

Soil suppressiveness is another important yet underutilized method for controlling soilborne pathogens. The term "disease suppressiveness" is commonly used to describe soils or substrates, in which certain specific diseases are absent or occur only to a low degree, even though the pathogen is present and conditions are suitable for disease development. Baker and Cook (2) described suppressive soils as “soils in which a pathogen does not establish or persist, establishes but causes little or no damage, or establishes and causes disease for a while but thereafter the disease is less important, although the pathogen may persist in the soil.” In contrast, conducive soils (nonsuppressive) are soils in which disease readily occurs (56). Two different types of disease suppressiveness are known: long standing and induced. Long standing suppressiveness is naturally associated with the soil, its origin is not known, and is independent of crop history. In contrast, induced suppressiveness is dependent on agricultural practices such as monoculture (56).

Fluorescent *Pseudomonas* spp. are common inhabitants of the rhizosphere and have been the focus of many studies as biological control agents (15, 39, 55, 56). Fluorescent *Pseudomonas* spp. synthesize a wide variety of antibiotics that are produced in the rhizosphere and contribute to pathogen suppression: phenazines, 2,4-diacetylphloroglucinol (DAPG), pyoluteorin, pyrrolnitrin, lipopeptides, and hydrogen cyanide (14). DAPG-producing *P. fluorescens* are some of the most effective biocontrol agents. DAPG is a key determinant in the biocontrol of root and seedling diseases: root rots of tobacco and tomato, Pythium damping-off of cucumber and take-all by strain CHA0 (8, 19, 44); damping-off of sugarbeet and cyst nematode and soft rot of potato by strain F113 (6, 7, 45); and take-all by strains Q2-87, Q8r1-96, and SSB17 (38, 47, 41).

Take-all, caused by the fungus *Gaeumannomyces graminis* var. *tritici*, is an important disease of wheat. Take-all is controlled by crop rotation, but it is also suppressed by continuous monoculture following an outbreak of the disease. This is known as take-all decline (TAD), and like take-all occurs worldwide. *P. fluorescens* producing the antibiotic DAPG were shown to be responsible for take-all suppression in TAD fields in Washington State and The Netherlands (41, 47). DAPG producers appear to be responsible for TAD in other wheat growing regions in the U. S. In order for take-all to be suppressed, population densities of DAPG producers must reach a threshold density of 10^5 CFU/g of root (41).

Enrichment of DAPG producers by continuous crop monoculture is not limited to cereals and may commonly contribute to the defense of plant roots against soilborne pathogens (56). For example, population densities of DAPG-producing *P. fluorescens* were above the threshold level (10^5 CFU/g of root) required for disease suppression on

roots of wheat and flax grown in a soil from Fargo, ND that had undergone 103 years of continuous flax monoculture. However, DAPG producers were not enriched in an adjacent field that had undergone over a century of crop rotation (23). In addition, wheat and pea grown in soil from Mount Vernon, WA, which had a >30 year history of continuous pea monoculture and is known to be suppressive to Fusarium wilt of pea (18), supported populations of DAPG-producing *P. fluorescens* in the rhizosphere above the threshold (22).

To date, 22 distinct genotypes (A-T, PfY and PfZ) of DAPG-producing *P. fluorescens* have been described on the basis of molecular fingerprinting methods and phylogenetic analysis (11, 22, 23, 27, 31). Although multiple genotypes often occur in a soil, the crop species exerts a selective pressure leading to the dominance of one or two genotypes that can aggressively colonize the rhizosphere of that crop (23). The crop species also affects the competitive interactions among genotypes in the rhizoplane (10, 29).

Given that monoculture soils of other crops enriches for DAPG producers, we hypothesize that those monoculture soils should also be take-all suppressive. The objectives of this experiment were: i) to assess the population dynamics of indigenous DAPG-producing *P. fluorescens* in monoculture and non-monoculture soils and the effect of long-term storage on those populations, ii) to determine the ability of introduced *P. fluorescens* strains of different DAPG-genotypes to survive in soil, and iii) to evaluate the ability of indigenous DAPG producers in a monoculture pea field soil to suppress take-all of wheat.

MATERIALS AND METHODS

Soils. Soils were collected with a shovel from the upper 30 cm of the soil profile from agricultural and undisturbed fields over the last 24 years; the history of each soil is described in Table 1. Soil samples were placed in plastic buckets with lids, transported to Pullman WA, air-dried, and stored outside under a shelter with no temperature control. Temperature in Pullman normally ranged as between -6°C in winter to 38°C in summer. Before being used, soils were sieved through a 0.5 cm mesh.

Bacterial growth media. Population sizes of total culturable heterotrophic bacteria in the rhizosphere were determined on 1/10x tryptic soy broth supplemented with cycloheximide (100 µg/ml) (TSB^{+}). Population sizes of indigenous DAPG-producing *P. fluorescens* were determined on a modified semi-selective medium for fluorescent pseudomonads, which consisted of one-third strength King's B medium supplemented with ampicillin (40 µg/ml), cloramphenicol (13 µg/ml), and cycloheximide (100 µg/ml) (1/3x KMB^{+++}) (22).

Wheat cycling to activate microbial populations in soils. In order to activate microbial populations in the soils, 20 wheat seeds (cv. Penawawa) were sown in three pots containing 1.5 kg of soil. At sowing, 200 ml of water was added to the soils and the pots were covered with a plastic film for 5 days in order to maintain high humidity and prevent the soil from drying before seedling emergence. After the plastic was removed, pots were watered at 3-day intervals, and twice a week received 100 ml of a fertilizer solution 15-30-15 (0.93 g/L) (Scotts Miracle-Gro Products, Port Washington, NY). Plants were maintained in a greenhouse at 15°C and under a 12 h photoperiod. Three weeks

after planting, plants with roots were carefully removed from the pots. Two plants were randomly selected from each pot and the roots with adhering soil were excised for detection of DAPG-producing *P. fluorescens* as described below. The remaining roots were cut into small pieces and added back into the soil, which was returned to the pots and wheat seeds were sown again to begin the next three-week cycle of growth.

Determination of rhizosphere colonization by DAPG-producing *P. fluorescens*. Roots with adhering rhizosphere soil were sampled at the end of each growth cycle of three weeks. Roots with adhering rhizosphere soil were excised from the shoot, placed in 50-ml screw-cap centrifuge tubes and 10 ml of sterile distilled water was added. The samples were shaken for 1 min on a Vortex mixer, and then sonicated in an ultrasonic cleaner (Bransonic 521, Branson, Shelton, CT) for one minute. The number of DAPG-producing bacteria was estimated using the PCR-based dilution end-point assay essentially as described by Landa et al. (22). Aliquots (100 µl) of the wash solution were serially diluted (1:3) in a 96-well microtiter plate pre-filled with 200 µl of sterile distilled water per well. The density of total culturable hetrotrophic bacteria in each sample was determined by transferring aliquots of 50 µl from the serially diluted (1:3) root washings into a 96-well microtiter plate containing 1/10x TSB^{+}. Microtiter plates were incubated at room temperature in the dark and bacteria growing in 1/10x TSB^{+} were assessed spectrophotometrically (Dynatech, MR5000, Dynatech Laboratories, Burlington, MA) after 48 ± 4 h. The terminal dilution in the microplate showing positive growth ($OD_{600} \geq 0.07$) was used to calculate the total population of culturable bacteria in a sample.

An aliquot of 50 µl of each dilution also was transferred to a well of a 96-well plate containing fresh 1/3x KMB^{+++} broth. Microtiter plates with 1/3x KMB^{+++} were

incubated at room temperature in the dark and bacterial growth was assessed spectrophotometrically after 96± 4 h. An absorbance at 600 nm (OD_{600}) of ≥ 0.07 was scored as positive for growth. The terminal dilution culture (TDC) is the greatest dilution (well) showing bacterial growth. Aliquots from wells showing growth were tested for the presence of DAPG-producing *P. fluorescens* by PCR as described below, and population densities were determined on the basis of the last well where the *phlD* signal was detected.

PCR amplification and detection of *phlD*. The PCR-based dilution end point assay was used to enumerate population densities of DAPG-producing *P. fluorescens* in the rhizosphere of wheat grown in cropped, non-cropped soils and soils in experiments designed to detect take-all suppressiveness. Aliquots from TDC of KMB^{+++} microtiter plates were subjected to PCR amplification to detect the *phlD* gene. Before PCR amplification, entire microtiter plates were frozen at -80°C for a minimum of one hour and PCR reactions were done essentially as was described previously (22, 30). Amplification was carried out in a 25 µl reaction volume containing 2.5 µl of thawed whole cell template. Amplifications were performed with a PTC-200 thermal cycler and the resulting PCR products were separated in 1.5% agarose gel in 0.5x tris-borate-EDTA (TBE) buffer at 125 V for 2 h. Banding patterns were visualized by ethidium bromide staining and scored by comparison to a 1 Kb bp ladder.

Production of *G. graminis* var. *tritici* inoculum. The inoculum of *G. graminis* var. *tritici* isolate R3-111a-1 (57) was prepared as described previously (30, 37). Briefly, 250 ml of distilled water and 250 ml of oats in a 1 liter flask were autoclaved twice on different days; then, one-half of a petri plate of 1/5x PDA (40 g potatoes, 5 g dextrose, 18

g agar 1 L distilled water) with a 7-day-old culture of *G. graminis* var. *tritici* was cut into pieces and mixed with the oats. The pathogen was allowed to colonize the oats for three to four weeks at room temperature in the light. Colonized oat grains were then air dried in a laminar flow hood, stored in paper bags at 4°C, and then pulverized in a blender immediately prior to use. Particles from 0.25 to 0.5 mm were added to the soil as the source of *G. graminis* var. *tritici* inoculum.

Evaluation of take-all suppressiveness in wheat and pea monoculture soils. Suppressive soils from fields in Quincy (Quincy TAD) and Mount Vernon (MV) known to have high population sizes of DAPG producers were used to further examine the role of DAPG producers in the suppression of take-all of wheat (Table 2). A conducive soil from Quincy (Quincy virgin) was used as a control because DAPG producers were not detected in that soil during the process of microbial activation described above. Once population sizes of DAPG producers in the suppressive soils were determined to be above log 5 CFU/g of fresh root, inoculum of *G. graminis* var. *tritici* was introduced into the soils at a rate of 0.5 % (w/w)**.** Six square polyvinyl chloride pots (8 cm high, 7.5 cm wide) were filled with 200 g of inoculated soils and then ten wheat seeds (cv. Penawawa) were sown into each pot. After sowing, 40 ml of water supplemented with metalaxyl (Novartis, Greensboro, N. C.) at 2.5 mg of active ingredient/ml were added to control Pythium root rot caused by indigenous *Pythium* spp. Pots were covered with a plastic film for 5 days to maintain humidity and to allow the seeds to germinate. After removal of the plastic film, pots were watered at 3-day intervals and twice a week received 40 ml of a fertilizer solution 15-30-15 (0.93 g/L) (Scotts Miracle-Gro Products, Port Washington, NY). The experiment was arranged in a completely randomized design.

Each treatment was replicated six times, and each pot served as a replicate. Plants were maintained in a greenhouse at 15°C with a 12 h photoperiod; after 3-weeks of growth, roots were carefully removed. The root system of one plant from each of the replicate pots was randomly selected for detection of DAPG-producing *P. fluorescens.* Six plants were randomly selected from each pot to determine take-all severity and the remaining roots were cut in small pieces and added back into the soils. Soils were returned to the pots and wheat seeds were sown again to begin the second cycle of growth. Take all disease severity was scored visually on a 0 to 8 scale, where 0 is a healthy plant and 8 is a dead plant (37). Shoot height and shoot fresh weight were also recorded in six randomly selected plants from each replicate to assess effect of take-all disease.

Transfer of suppressiveness. The transferability of disease suppressiveness was tested by diluting (1:9) Mount Vernon, Quincy TAD or Quincy virgin soils into steamed pasteurized Quincy virgin soil (Table 2), essentially as described by Raaijmakers and Weller (41). After thoroughly mixing the soils, inoculum of the take-all pathogen was introduced. Six square polyvinyl chloride pots (8 cm high, 7.5 cm wide) were filled with 200 g of inoculated soils. Each pot was amended with 40 ml of water supplemented with metalaxyl (Novartis, Greensboro, N. C.) at 2.5 mg of active ingredient/ml to control Pythium root rot caused by indigenous *Pythium* spp. Ten seeds of wheat (cv Penawawa) were planted in each pot. The experiment was arranged in a completely randomized design. Each treatment was replicated six times, and each pot served as a replicate. After 3 weeks of growth, plants and roots were harvested. Plants were rated for take-all disease, and shoot height and shoot fresh weight was recorded, as described above.

Effect of pasteurization on disease suppression in the Mount Vernon soil. Soil from Mount Vernon was subjected to pasteurization (60°C, 30 min) (Table 2), which is known to eliminate take-all suppressiveness and DAPG producers (41). After the pasteurization process, the soil was allowed to cool and dry. Inoculum of *G. graminis* var. *tritici* was added into the soil at a rate of 0.5 % (w/w), and then ten wheat seeds (cv. Penawawa) were sown. Natural Mount Vernon soil harboring DAPG producers above log 5 CFU/g of fresh root amended with *G. graminis* var. *tritici* inoculum was used as a control. Each treatment was replicated six times and each pot (square polyvinyl chloride pots 8 cm high, 7.5 cm wide) served as a replicate. After 3 weeks of growth, plants and roots were harvested. Take-all disease on plants was determined as described above.

Population densities of DAPG-producing *P. fluorescens* and total culturable aerobic heterotrophic bacteria in the rhizosphere of wheat infected by *G. graminis* var. *tritici.* Roots from six randomly selected plants from each of the treatments in the experiments, including undiluted, diluted and pasteurized soils in which the take-all pathogen was introduced, were used to determine population densities of DAPG-producing *P. fluorescens* and total culturable heterotrophic bacteria, as described above.

Long term survival of DAPG-producing *P. fluorescens* genotypes in soil. In a cycling experiment originally initiated by Landa et al. (21), strains CHA0 (genotype A), Pf-5 (genotype A), Q2-87 (genotype B), 1M1-96 (genotype L), W2-6 (genotype D), L5.1-96 (genotype D), Q8r1-96 (genotype D) and Q8r2-96 (genotype D) were introduced individually into a natural Quincy virgin soil in a suspension of 1% methylcellulose to give approximately 10^4 colony forming units (CFU)/g fresh weight of soil. At the end of the experiment, which lasted for 10 cycles of wheat (3 weeks each), Landa et al. (21)

placed the soils in open plastic bags, allowing them to air dry, and then stored them at room temperature (24°C). To assess the long-term survival ability of different DAPG genotypes, one year later, the stored soils were placed in pots, each pot was filled with 200 g of soil, and eight wheat seeds were sown. Pots received 50 ml of water supplemented with metalaxyl (Novartis, Greensboro, NC) at 2.5 mg/ml active ingredient after sowing to control Pythium root rot. Pots were completely randomized on the greenhouse bench, covered with a plastic film and incubated for 72 h at 15^{o}C to maintain a high relative humidity until the seedlings emerged. Each treatment consisted of two pots, with each pot serving as a replicate. Plants were grown in a greenhouse at 15±1^{o}C, with a 12-h photoperiod. Pots were watered at 2-day intervals and twice a week received 50 ml of a Miracle-Gro solution 15-30-15 (Scotts Miracle-Gro Products; Port Washington, NY). Three plants were selected randomly from each pot at the end of each cycle in order to determine the population size of the introduced bacteria. Shoots of the remaining plants were excised, and the soil and associated root systems from the pots of the same treatment were decanted into a plastic bag and mixed by shaking. The soil was returned to the pots and immediately replanted with eight wheat seeds, beginning the next cycle. The process of growth, harvesting, and determination of population sizes was repeated in growth cycles of 3-weeks each. Population densities of total culturable heterotrophic bacteria were determined in each growth cycle as described above. Since introduced DAPG-producing *P. fluorescens* strains were rifampicin-resistant mutants, population densities were assessed by using 1/3x KMB^{+++} amended with 100 µg/ml of rifampicin.

At the end of cycle 5, soils were again air-dried, placed in plastic bags and stored at room temperature. After 5 months of storage, the cycling process, as described above, was initiated again. However, in this second period of cycling, a portion of the stored soils was diluted (1:9) with pasteurized Quincy virgin soil in order to assess the ability of the DAPG producer strains to be transferred into a pasteurized soil and colonize the rhizosphere of wheat. The remaining original soils were also cycled. After 3 cycles of growth, original and diluted soils were again air-dried, placed in plastic bags and stored at room temperature (24°C). A final period of wheat cycling was initiated after the soils had been stored for 8 months only in the soils were the DAPG producers were originally introduced by Landa et al. (21). Population densities of total culturable heterotrophic bacteria and DAPG producers in the wheat rhizosphere were determined as described above.

Determining the genotype of DAPG producers in the long-term survival experiment. To determine the identity of specific DAPG genotypes, RFLP analyses were conducted from the terminal p*hlD*$^{+}$-amplified PCR products of rhizosphere populations. Aliquots (8 µl) of a PCR reaction were digested in a total volume of 30 µl with 10 units of the *Hae*III restriction enzyme as previously described (22). Reactions were incubated for 3 h at 37°C. Restriction digestion with this enzyme can clearly differentiate the D and K genotypes present in the soils. All products were stored at –20°C. Digested products were separated on 2% agarose gels in 0.5x TBE buffer for 3 h at 140 V. Banding patterns were visualized by ethidium bromide staining and scored by comparison to a 100 bp ladder.

Data analysis. All experiments were arranged in a completely randomized design. The experiments of take-all suppression were performed at least 2 times. In some cases, representative results of one experiment are shown. All population data were converted to Log CFU/g fresh weight of root. Data were analyzed using STATISTIX (version 8.0, Analytical Software, Tallahassee, FL). Differences between treatments in population densities, shoot height and shoot fresh weight were determined by standard analysis of variance, and mean comparisons among treatments were performed using Fisher's protected least significant difference test at P=0.05. When shoot height or fresh shoot weight was not normally distributed, a rank transformation was conducted before statistical analysis. Differences between treatments in take-all severity were analyzed by using the non-parametric Kruskal-Wallis test.

RESULTS

Population densities of indigenous DAPG-producing *P. fluorescens* in monoculture and non-monoculture soils. The wheat cycling process was used to determine the population densities of DAPG producers in the rhizosphere of wheat grown in each of ten different soils (Table 1). This is the first time that all of these soils were compared simultaneously.

Population densities of DAPG producers in the rhizosphere of wheat grown in the non-cropped soils and in the Fargo crop rotation soil were below the detection level (Log 3.26 CFU/g root) during all four cycles of wheat growth (Fig. 1). In contrast, in the

rhizosphere of wheat grown in the soils that had undergone continuous crop monoculture (flax, pea, or wheat), population densities of DAPG producers were above the threshold required for disease suppression (log 5 CFU/g of root) (Fig. 1) beginning in the first, second or third cycle of growth. Population densities of DAPG producers increased during cycling most rapidly in the Quincy TAD and Mount Vernon soils, which were the most recently collected soils. Even though the Lind and Moses Lake TAD soils were stored untouched in cans since 1982 and 1986, respectively, the population dynamics of the DAPG producers in the rhizosphere during cycling were the same as in the other monoculture soils.

Soils from Fargo, ND represent very different soil type than those in the Pacific Northwest, yet the population dynamics of the DAPG producers in the rhizosphere were similar as in the Washington state soils. It should be noted that the fields that serve as the source of the Fargo soils were side-by-side with the crop rotation field in between the two crop monoculture fields.

Take-all suppressiveness in pea and wheat monoculture soils. Soil from the pea monoculture field was compared to Quincy TAD soil for ability to suppress take-all. Prior to the test for take-all suppression, both soils were cycled to wheat to insure that the population densities of DAPG producers were above the threshold level required for disease suppression (log 5 CFU/g of root).

The severity of take-all on wheat plants grown in Quincy TAD or Mount Vernon soil was significantly less (P=0.05) as compared to the disease severity on plants grown in the conducive Quincy virgin soil (Figs. 2, 4), based on measurements of several parameters including disease severity, shoot height and shoot fresh weight. When Quincy

TAD, Mount Vernon and Quincy virgin soils were diluted (1:9) with a steamed pasteurized conducive soil (Quincy virgin), significantly (P=0.05) less take-all occurred in the diluted suppressive soils than in the diluted conducive soil (Fig. 3).

Population densities of total culturable aerobic heterotrophic bacteria and DAPG-producing *P. fluorescens* in the rhizosphere of wheat infected by *G. graminis* var. *tritici*. Population sizes of indigenous DAPG-producing P. *fluorescens* were greater than log 6 CFU/g fresh root in soils with a history of wheat and pea monoculture (Fig. 5), whereas populations of DAPG producers were below the detection level in the Quincy virgin soil (Fig. 5) and pasteurized soils (Table 4). Populations of DAPG producers above log 6 CFU/g fresh root occurred in the rhizosphere of wheat grown in monoculture soils diluted (1/:9) with steamed pasteurized conducive soil (Quincy virgin) (Table 4), thus showing the ability of the indigenous DAPG- producing *P. fluorescens* to aggressively colonize a pasteurized conducive soil.

Population sizes of total culturable aerobic heterotrophic bacteria were near log 9 CFU/g fresh root, and did not differ significantly among the soils (data not shown).

Effect of pasteurization on disease suppressiveness and population density of DAPG producers in the pea monoculture soil. Mount Vernon suppressive soil subjected to pasteurization (60°C, 30 min), no longer harbored detectable populations of DAPG producers (Table 4). Take-all severity was significantly greater (P=0.05) on wheat grown in the steamed pasteurized Mount Vernon soil as compared to the non-pasteurized soil during cycles 1 and 2 in both experiments, based on measurements of severity of the disease, plant height and shoot fresh weight (Table 4).

Population densities of total culturable aerobic heterotrophic bacteria in the rhizosphere of wheat grown in soils infested with *G. graminis* var. *tritici* were greater than log 8 CFU/g fresh root in the first and second cycles of growth and did not differ in the rhizosphere of wheat in the pasteurized and non-pasteurized soils (Table 4).

Survival in soil of DAPG-producing *P. fluorescens* genotypes. In a previous experiment (21), DAPG-producing *P. fluorescens* strains were introduced into the soil at a rate of 10^4 CFU/g of soil. In that experiment, population densities of the DAPG producers in the wheat rhizosphere were monitored for 10 cycles of three weeks each. These soils were used to further assess the survivability of DAPG producers. Soils were then air dried and stored at room temperature for one year.

After one year of storage, these soils were sown to wheat and population densities of DAPG producers were detected only in the rhizosphere of wheat (cv. Penawawa) grown in soils containing strains F113 (genotype K) and L5.1-96 (genotype D). As a result, those two soils were selected for further study. During the first five wheat cycles after the soil was stored for one year, population densities of total culturable aerobic heterotrophic bacteria in soils containing L5.1-96 and F113 strains were significantly different only in the 5^{th} cycle (P=0.001) (Table 5). Population densities of strains F113 (genotype K) and L5.1-96 (genotype D) in the rhizosphere of wheat were above log 5 CFU/g fresh root in all cycles. In the first cycle, population densities of both strains in the wheat rhizosphere did not differ significantly (P=0.99); however, population densities of strain L5.1-96 were significantly (P=0.001) greater as compared to strain F113 during all remaining cycles (Fig. 8). At the end of cycle number five, samples of soil free of roots from each treatment were analyzed to quantify the population densities of DAPG

producers. Population densities of both strains in the soil did not differ significantly (P=0.05) (Fig. 6).

At the end of the 5th wheat cycle, soils were air dried and stored for five months. At the beginning of the new period of wheat cycling, a volume of the soil containing strains L51-96 or F113 was diluted (1:9) with steamed pasteurized Quincy soil to assess the ability of the strains to colonize a conducive soil and the rhizosphere of wheat. The remaining undiluted or original soil also was cycled. Wheat was grown for three cycles of 3-week each. Population densities of total culturable aerobic heterotrophic bacteria ranged between log 8.1 and log 9.34 CFU/g of fresh root in the original soils (Table 5), and log 8.00 to log 9.50 in the diluted soils (Fig. 7). Population densities of strain F113 were below the detection level (log 3.26 CFU/g of fresh root) in the rhizosphere of wheat grown either in the original (Fig.8) or the diluted soil (Fig. 7).

Strain L5.1-96 was able to colonize the rhizosphere of both original undiluted and diluted soils. In the three cycles of wheat, populations densities of L5.1-96 were detected at levels above the threshold required for disease suppression (log 5 CFU/g fresh root) in both original (Fig. 8) and diluted soils (Fig. 7).

At the end of the third cycle of wheat growth, soils were again air dried, placed in plastic bags, and subjected to a new period of storage of 8 months. After this period of storage, DAPG-producing *P. fluorescens* strain L5.1-96 was able to colonize the rhizosphere of wheat and be detected by the PCR-based dilution end-point assay. The greatest population density of strain L51-96 was log 4.54 CFU/g of fresh root detected at the end of the second cycle (Fig. 8). Soils originally containing strain F113 were also

stored under the same conditions; however, population densities of strain F113 were below the detection level in all of the cycles (Fig. 8).

Strain L5.1-96 was able to survive in soil at room temperature, for cumulative periods of time comprising three storage periods of one year, five months, and eight months (Fig. 8). This is the first time that population densities of an introduced biological control strains are reported over an extended period of time.

DISCUSSION

In this study, we determined the population dynamics of indigenous DAPG-producing *P. fluorescens* in ten different soils from undisturbed (virgin) sites, and from fields that had undergone continuous crop monoculture or crop rotation. The process of wheat cycling was conducted to activate the existing soil microflora in a uniform manner so that the DAPG producers in each soil could be compared. Wheat is used for cycling because it supports colonization by all know genotypes of DAPG producers. The soils were collected at different dates, ranging from 2 to 22 years ago, and were maintained dried either at room temperature or outside in plastic or metal buckets.

Population densities of DAPG-producing *P. fluorescens* above the threshold for suppression were only found in the rhizosphere of wheat grown in soils that had undergone wheat, flax or pea monoculture. Although DAPG producers were previously isolated from these soils (21, 23, 31, 41), this is the first time that the survival and persistence of indigenous DAPG producers in these soils was compared in a single study.

In contrast, in the rhizosphere of wheat grown in non-cropped soils or soils that had undergone crop rotation, DAPG producers were not found in any of the wheat cycles conducted (Fig. 1). The role of populations of DAPG-producing *P. fluorescens* in take-all suppression in the Quincy, Lind, and Moses Lake TAD soils and also in suppression of Fusarium wilt in the Mount Vernon pea monoculture soil has been previously reported (42; B. B. Landa and D. M. Weller unpublished data). However, the role of the indigenous DAPG producers in the Fargo flax and wheat monoculture soils in disease suppression is a topic of current investigation.

A considerable amount of research has been conducted during the last three decades on the growth and survival of introduced rhizobacteria in the soil and rhizosphere. In general, bacterial populations applied as biocontrol agents undergo a steady decline in population size once introduced into the soil (35, 51, 52), and this is one reason given for the failure of disease suppression (54). The plant species or cultivar, the composition of root exudates and the type of soil and agricultural management play an important role in the composition of the bacterial community that colonize and survive in the rhizosphere (3, 4, 10, 12, 24, 25, 29, 46). In this study, the availability of monoculture soils that had been stored for up to 22 years allowed for the first time an assessment of the persistence and reactivation of indigenous naturally-ocurring DAPG producers. In most cases, populations of indigenous DAPG producers are below the detection level, and in our study DAPG producers were not detected in the bulk soil by using the PCR-based dilution endpoint assay (data not shown). However, once wheat was grown, populations of DAPG-producing *P. fluorescens* were re-activated and easily detected in the wheat rhizosphere, and by the end of the third cycle of wheat were above the

threshold level of disease suppression (log 5 CFU/g fresh root) (41). We were surprised to find that the DAPG producers in the Lind, Moses Lake and Fargo soils responded so rapidly to the presence of wheat roots after storage of the soils for 22, 18, and 6 years, respectively. We think that the process of soil monoculture alters the population of DAPG producers and "primes" it in some manner that allows it to rapidly respond when a crop is sown and roots push through the soil. This possibility is supported by the finding than indigenous DAPG producers in virgin soils do not respond and increase during cycling in the same manner (41).

The availability of soils samples from a previous experiment by Landa et al., (21) in which strains of five different DAPG genotypes had been introduced into the soil at log 4 CFU/g of soil, cycled to wheat, and the soil then dried, allowed for the further assessment of the long-term survival of DAPG-producing *P. fluorescens*. Of the five genotypes of DAPG producers tested only *P. fluorescens* strains L5.1-96 (genotype D) and F113 (genotype K) could be detected in dry soil after one year of storage. However, only strain L5.1-96 persisted through a succession of wheat cycling and storage in dry soil. It is notable that strain L5.1-96 originally was isolated from wheat roots grown in the Lind TAD soil (21), which was the same TAD soil showing the survival of indigenous populations of *P. fluorescens* for over 22 years after being collected. In total, these studies indicate that the genotypes and individual strains differ significantly in ability to survive for long periods of time in soil in the absence of plants. Survival ability cannot be linked to specific genotypes since Q8r1-96, Q8r2-96 and L5.1-96 all belong to the D genotype and are from TAD soils yet showed differential abilities to persist in dry soil.

Other studies have addressed the persistence of introduced *Pseudomonas* spp. in soil. For example, a rifampicin-resistant mutant of *P. fluorescens* strain NBRI2625R, which was isolated from a disease suppressive soil in India, could not survive in the field for 7 months (36). *P. fluorescens* strain BBC6R8 was not detected four years after being introduced into a Douglas fir forest plantation (16). Survival in soil for less than one year was reported for *P. putida* (34). Under field conditions, the population density of *P. fluorescens* strain F113 was log 2 CFU/g of root system in clover, one year after the strain had been introduced at log 6 CFU/seed of sugarbeet (33). Dupler and Baker (9) showed that *Pseudomonas putida* introduced at a rate of log 7 CFU/g of soil was able to survive in a fallow soil for over 10 months.

The basis of the enrichment of populations of DAPG producers in monoculture soils is a topic of considerable research interest, and may involve the continual availability of certain key exudate(s). Our observation that indigenous DAPG producers were persistent in the soil for over 22 years and they still were able to colonize the roots of wheat represent an unique ability of this group of biocontrol bacteria. *Pseudomonas* spp. are non-spore forming bacteria, thus it is surprising their ability to persist in soil and then rapidly rebound in populations when a suitable host is grown, and it is a topic of current investigation.

The importance of the persistence of biocontrol agents in the rhizospehere has been emphasized (56) as essential for successful biocontrol; however, only a few studies have assessed the survival of introduced microorganisms in soil. Usually, survival studies have focused on risks assessment studies for introduced genetically engineered

microorganisms and their ecological impact on microbial populations or the ability of introduced strains to survive within a crop cycle of growth (13, 49).

One key question is how DAPG-producing *P. fluorescens* populations survived in soil for these long periods of time? Exhaustion of nutrients, predation by protozoa, competition, cell death or formation of viable but non culturable cells are among the factors contributing to the decline of indigenous and introduced microorganisms into the soil (52). Among biotic factors that can influence survival and colonization ability of *Pseudomonas* spp. is the presence in soil of virulent bacteriophages. Keel et al. (20) showed that a lytic bacteriophage reduced the root colonization ability and the biocontrol efficacy of *P. fluorescens* CHA0 in a natural soil.

Under non-favorable conditions many bacteria, including *P. fluorescens* strains, enter a viable but nonculturable (VBNC) state (5, 52); however, the ecological significance of the VBNC state to biocontrol bacteria has still not been elucidated. For instance, Mascher et al., (26) noted that *P. fluorescens* CHA0 was able to form VBNC cells as a response to low redox potential, oxygen limitation and different NaCl levels in vitro. However, 54 days after being introduced into the soil, *P. fluorescens* CHA0 cells in a VBCN state did not display enhanced persistence as compared with culturable cells. In a different study, after 40 days in soil microcosms only 0.02%-0.35% of the initial population of *P. fluorescens* DF57 was culturable, however 20% of the initial inoculum introduced was observed to be viable but non-culturable cells (5). The undetectable level of populations of DAPG producers in the monoculture soils before the cycling of wheat probably was due to the *P. fluorecens* cells being in the VBNC state; however, with

wheat cycling, cells convert from this state to an active state in which they recover their colony-forming ability.

Survival of introduced bacteria also has been related to soil characteristics including soil texture and structure. van Elsas (51) observed a decline in populations of *P. fluorescens* WCS374 initially introduced at $3.5x10^7$ cells/g soil to between 10^4 and 10^6 cells/g of soil in silt loam and below $1x10^2$ in a loamy sand soil after 120 days in un-cropped conditions. *P. fluorescens* survived better in the silt loam soil, which has a finer texture than the coarser sandy soil. Survival of soils in finer textured soils has been related with greater availability of suitable niches represented by micropores in the clay soils, where bacteria could also be protected against bacterial-eating protozoans (59).

DAPG-producing *P. fluorescens* have been detected in soils worldwide that are suppressive to root and wilt diseases of wheat, barley, tobacco and pea (22, 41, 43, 47). The role of indigenous DAPG producers in the suppression of take-all in soils under continuous wheat monoculture (TAD soils) has been conclusively demonstrated (42, 47). We hypothesized that soils with populations of DAPG producers enriched by monoculture of other crops should also be suppressive to take-all. To test this hypothesis we used a Puget silt loam soil from a field in Mount Vernon, WA that had been under pea monoculture for over 30 years and is known to be suppressed to Fusarium wilt caused by *Fusarium oxysporum* f. sp. *pisi* (18). We followed the approaches outlined by Raaijmakers and Weller (41), to test the role of DAPG producers in the suppressiveness. We showed that wheat grown in the QTAD and Mount Vernon soils had significantly less (P=0.05) disease than wheat in the conducive virgin soil. Furthermore, although populations of total aerobic heterotrophic bacteria were the same on the roots of all three

soils, threshold population densities of DAPG producers were detected only on the roots of wheat in the QTAD and Mount Vernon soils but not in the Quincy virgin soils. We demonstrated that suppressiveness was biologically based and not due to soil physical/chemical factors by transferring (diluting 1:9) each soil into a pasteurized Quincy virgin soil (Figure 3). Disease severity on wheat in the transferred pea monoculture soil was again similar to the level of disease in the transferred QTAD, and both showed significantly less (P=0.05) disease than wheat grown in the conducive transferred Quincy virgin soil. At the end of the experiment, population densities of total aerobic heterotrophic bacteria were equivalent on roots of wheat in all of the transferred soils, however roots in the Quincy TAD and Mount Vernon transferred soils had densities of DAPG producers >log 6 CFU/g of fresh root whereas DAPG producers were not detected in the transferred Quincy virgin soil.

Finally, pasteurization of the pea monoculture soil from Mount Vernon eliminated DAPG producers from the roots of wheat grown in the soil and resulted in greater disease on plants (Table 4) as compared with the disease severity observed in the non-treated Mt. Vernon soil. The density of total aerobic heterotrophic bacteria was the same on roots of wheat in the pasteurized and raw Mount Vernon soil. Loss of suppressiveness by pasteurization has been demonstrated in other soils containing antagonistic *Pseudomonas* spp. (1, 41, 48, 58).

Populations of indigenous DAPG producers on roots infected by the take-all pathogen were greater as compared with the population densities on healthy roots in the Quincy TAD soil. This phenomenon of increased populations of pseudomonads on roots with lesions have be reported previously (28, 30) and is likely due to the greater leakage

of root exudates and cell components from infected roots, and as a result more nutrients are available for the microbial community in the rhizosphere.

In conclusion, the present study shows new evidence that DAPG producers are enriched by continuous monocultures of crops other than wheat and barley (23, 56) and the functional role that those indigenous populations of DAPG producers have in the suppression of take-all. Our results confirm the correlation between threshold population sizes of DAPG producers in the rhizosphere of wheat and the reduction of take-all disease previously identified by Raaijmakers and Weller (41).

The suppression of take-all by the DAPG producers enriched through pea monoculture opens up a new view of suppressiveness in soils containing indigenous DAPG producers. The specific suppressiveness of a soil has been thought to be active against a single pathogen or closely related group of pathogens (56). However, under our experimental conditions we demonstrated that the suppressiveness of the monoculture pea soil from Mount Vernon known to be effective against Fusarium wilt on pea caused by *Fusarium oxysporum* f. sp. *pisi* (18) is also suppressive to take-all of wheat caused by *G. graminis* var. *tritici*. To our knowledge this is the first example of specific suppressiveness operating against two distinct diseases. The fact that *G. graminis* var *tritici* and *F. oxysporum* f. sp. *pisi* are sensitive to DAPG (3 and 15 μg/ml, respectively) is probably the basis of the common suppression.

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Table 1. Source of suppressive and conducive soils and cropping history.

Soil	Location of field	Cropping history	Years in monoculture[a]	Disease suppressiveness[b]	Date soil collected	References
Quincy virgin	Quincy, WA	Non-cropped[c]	0	-	2002	(42)
Quincy TAD	Quincy , WA	Wheat	22	+ (TAD)	2002	(42)
Mount Vernon	Mt. Vernon, WA	Pea	30+	+ (FWS)	2001	(22)
Lind virgin	Lind, WA	Non-cropped	0	-	1982	(42)
Lind TAD	Lind, WA	Wheat	28	+ (TAD)	2001	(42)
Moses Lake virgin	Moses Lake, WA	Non-cropped	0	-	1986	(42)
Moses Lake TAD	Moses Lake, WA	Wheat	22	+(TAD)	1986	(42)
Fargo flax	Fargo, ND	Flax	103	ND	1997	(23)
Fargo rotation	Fargo, ND	Crop rotation[d]	0	ND	1997	(23)
Fargo wheat	Fargo, ND	Wheat	115	ND	1997	(23)

[a] Number of years that fields had been in continuous wheat, pea or flax monoculture

[b] TAD= take-all decline; FWS= Fusarium wilt suppressive; ND= not determined,

[c] virgin sites with natives grasses and sagebrush,

[d] Field was rotated to a variety of crops (bean, corn, soybean, sugar beet, and sunflower, or left fallow) for >100 years.

Table 2. Soils used in the experiments of take-all suppression.

Soil	Treatment of soil	Addition of Ggt[c]
Natural soils		
Quincy virgin	cycled soil[a]	+
Quincy TAD	cycled soil	+
Mount Vernon	cycled soil	+
Transferred soils		
Quincy virgin T.	10 % cycled soil + 90 % pasteurized Quincy virgin soil[b]	+
Quincy TAD T.	10 % cycled soil + 90 % pasteurized Quincy virgin soil	+
Mount Vernon T.	10 % cycled soil + 90 % pasteurized Quincy virgin soil	+
Pasteurized soil		
Mount Vernon	Pasteurized cycled soil	+
Control soils		
Mount Vernon	cycled soil	-
Quincy TAD	cycled soil	-

[a] Soils were cycled at least 3 times prior to use in order to enhance population sizes of DAPG-producing *P. fluorescens.*

[b] Treated with aereated steam, 60°C for 30 min (41).

[c] Take-all pathogen (*G. graminis* var. *tritici*) was introduced as oat-kernel inoculum at a rate of 0.5 % (w/w).

Table 3. DAPG-producing *P. fluorescens* strains used in the long-term survival experiments.

Strain	Genotype[a]	Host crop[b]	log CFU/g fresh root[c]	Reference
Control	-------	-------		
Pf-5	A	Cotton	<DL[d]	(17)
CHA0	A	Tobacco	<DL	(48)
Q2-87	B	Wheat	<DL	(53)
F113	K	Sugar beet	7.2	(45)
1M1-96	L	Wheat	<DL	(40)
W2-6	D	Wheat	6.27	(31)
L5.1-96	D	Wheat	7.95	(21)
Q8r1-96	D	Wheat	6.19	(40)
Q8r2-96	D	Wheat	6.66	(31)

[a] Genotype based on previous determination by using BOX-PCR and restriction fragment length polymorphisms (RFLP) analysis of *phlD* (22, 27, 30). All strains used were spontaneous rifampicin-resistant mutants.

[b] Original source of the strain.

[c] Population densities detected in the wheat rhizosphere in the last 10th cycle prior to the initial storage (21).

[d]<DL= below the level of detection.

Table 4. Effect of pasteurization of the continuous pea monoculture soil on disease suppression of take-all of wheat.

		Cycle 1[a]					Cycle 2				
					log CFU/g fresh root					log CFU/g fresh root	
Soil	Ggt[b]	Disease rating [c]	Plant height (cm)	Shoot fresh weight (g)	TCB[d]	DAPG[e]	Disease rating	Plant height (cm)	Shoot fresh weight (g)	TCB	DAPG
Experiment 1											
MV[f]	-	0	22.0	0.38	8.13	6.36	0	21.0	0.33	8.37	7.11
MV	+	3.8b[h]	15.1a	0.23a	8.88b	8.56	2.5b	18.5a	0.34a	8.86a	7.23
MVP[g]	+	6.1a	12.9b	0.16b	9.20a	3.26[i]	5.4a	15.6b	0.24b	8.47a	3.26
P value		0.001	0.003	0.001	0.009		0.001	0.032	0.013	0.07	
Experiment 2											
MV	-	0	24.3	0.23	8.34	8.25	0	23.5	0.28	7.96	7.89
MV	+	3.3b	17.0a	0.15a	8.70a	7.63	2.5b	19.9a	0.20	9.10a	8.20
MVP	+	7.0a	12.0b	0.08b	8.56a	3.26	6.9a	12.4b	0.09	8.72a	3.26
P value		0.002	0.001	0.001	0.57		0.002	0.001	0.002	0.27	

[a]Each cycle consisted of 3 weeks of growth in the greenhouse at 15°C with a 12 h photoperiod.

[b]Inoculum of *G. graminis* var. *tritici* was introduced into the soil at a rate of 0.5 % w/w at the beginning of the first cycle. Each treatment consisted of six replicates. One pot containing 200 g of soil served as a replicate. 8 wheat seeds (cv. Penawawa) were sown per pot.

[c]The severity of take-all was determined by measuring disease on a 0-8 scale, plant height and shoot fresh weight. Values are the means of six replicates. Each replicate was the average of the measurement of six randomly selected plants within each replicate.

[d]TCB= Total culturable aerobic heterotrophic bacteria determined by 1/10x TSB^{+} . Values are the means of six replicates. One plant was randomly selected from each replicate.

[e]DAPG = DAPG-producing *P. fluorescens* detected using the PCR-based dilution end-point assay. Values are the mean of six replicates. One plant was randomly selected from each replicate.

[f]MV= Mount Vernon pea monoculture soil.

[g]MVP= Pasteurized Mount Vernon pea monoculture soil (60 °C, 30 min).

[h]Means in a column, within each experiment, followed by the same letter are not significantly different (P=0.05) according to Wilcoxon's rank sum test for take-all severity and two sample t-test for the remaining measured parameters.

[i]When DAPG-producing *P. fluorescens* were not detected, a value of log 3.26 CFU/g fresh root (detection limit) was assigned.

Table 5. Populations densities of total culturable heterotrophic bacteria in the wheat rhizosphere in long-term survival experiments[a].

	log (CFU g^{-1} root fresh weight)[b]				
	Cycle				
Strain	1	2	3	4	5
Experiment 1[c]					
F113	7.99a[f]	8.55a	8.92a	8.77a	7.87b
L5.1-96	7.66a	8.32a	8.55a	8.48a	8.43a
P value	0.098	0.093	0.79	0.64	0.001
Experiment 2[d]					
F113	8.10a	8.27b	9.08a		
L5.1-96	8.55a	9.20a	9.34a		
P value	0.184	0.001	0.128		
Experiment 3[e]					
F113	8.69b	8.74a	8.67a		
L5.1-96	9.30a	8.68a	8.67a		
P value	0.056	0.925	0.539		

[a] Total culturable heterotrophic bacteria in the wheat rhizosphere, determined by bacterial growth in 1/10x TSB^{+}.

[b] Values are the means of six replicate roots randomly selected from two pots in each treatment.

[c] Experiment 1= wheat growth cycles conducted after the soil from the Landa et al., (21) experiment was stored for one year.

[d] Experiment 2= wheat growth cycles conducted after the soil from experiment 1 was stored for five months.

[e] Experiment 3= wheat growth cycles conducted after the soil from experiment 2 was stored for eight months.

[f] Mean values with the same letter within a column in an experiment are not significantly different (P=0.05) according to a two-sample t-test.

Figure 1. Population densities of DAPG-producing *P. fluorescens* in soils with different cropping history. Wheat (cv. Penawawa) was grown in each soil (described in Table 1) for four cycles of 3-week each. Bars represent the population density of DAPG-producing *P. fluorescens* in the rhizosphere of six wheat plants randomly selected at the end of each cycle. When DAPG producers were not detected, the mean was assigned a value of log 3.26 CFU/g of root, which is the detection limit of the PCR-based end-point dilution assay.

Figure 2. Suppression of take-all in Mount Vernon continuous pea monoculture soil that is suppressive to Fusarium wilt. The severity of take-all was determined by measuring disease on a 0-8 scale, shoot height, and shoot fresh weight. Values are the mean of six replicates. Each replicate was the average of measurements of six plants and roots randomly selected. Bars within a panel with the same letter are not significantly (P=0.05) different according to Kruskal-Wallis test for take-all severity, and Fisher's protected least significant difference for shoot height and shoot fresh weight. The control treatment was not inoculated with *G. graminis* var. *tritici* and was not included in the statistical analysis. QV= Quincy virgin soil; QTAD= Quincy TAD soil; MV= Mount Vernon soil. Column 1 (Figs. 2 A, C, and E) and column 2 (Figs. 2 B, D and F) show two separate experiments.

Figure 3. Suppression of take-all in the Mount Vernon continuous pea monoculture soil that was diluted (1:9) with pasteurized Quincy virgin soil. The severity of take-all was determined by measuring disease on a 0-8 scale, shoot height, and shoot fresh weight.

Values are the mean of six replicates. Each replicate was the average of measurements of six plants and roots randomly selected. Bars within each panel with the same letter are not significantly (P=0.05) different according to Kruskal-Wallis test for take-all severity, and Fisher's protected least significant difference for shoot height and shoot fresh weight. The control treatment was not inoculated with *G. graminis* var. *tritici* and was not included in the statistical analysis. QVT= Quincy virgin soil diluted (1:9) with pasteurized Quincy virgin soil; QTADT= Quincy TAD soil diluted (1:9) with pasteurized Quincy virgin soil; MVT= Mount Vernon soil diluted (1:9) with pasteurized Quincy virgin soil. Column I (Figs. 2 A, C and E) and column 2 (Figs. 2 B, D and F) show two separate experiments.

Figure 4. Suppression of take-all in Quincy TAD and Mount Vernon soils. Inoculum of *G. graminis* var. *tritici* was introduced at a rate of 0.5 % (w/w). Each treatment was replicated six times and the experiment was repeated twice. Wheat seedlings are three weeks old. QV= Quincy virgin soil, QTAD= Quincy TAD soil, and MV= Mount Vernon soil.

Figure. 5. Population densities of DAPG-producing *P. fluorescens* in undiluted and diluted suppressive and conducive soils amended with take-all inoculum. Bars represent the means of six roots randomly selected. Standard deviations of the means are presented. Experiment was repeated twice, and results from a representative experiment are shown. QV= Quincy virgin soil; QTAD= Quincy TAD soil; MV= Mount Vernon soil; QVT= Quincy virgin transferred soil, (Quincy virgin soil was diluted [1:9] with pasteurized

conducive Quincy virgin soil); QTADT= Quincy TAD transferred soil, (Quincy TAD soil was diluted [1:9] with pasteurized conducive Quincy virgin soil); MVT= Mount Vernon transferred soil, (Mount Vernon soil was diluted [1:9] with pasteurized conducive Quincy virgin soil); MVP= steamed pasteurized Mount Vernon soil; MVC= Mount Vernon control (*G. graminis* var. *tritici* inoculum was not introduced in this soil), and QTADC= Quincy TAD control (*G. graminis* var. *tritici* inoculum was not introduced in this soil).

Figure 6. Population densities of DAPG-producing *P. fluorescens* strains F113 and L5.1-96 in soil after five 3-week cycles of wheat. Values are the mean of six samples of soil (0.5 g each) free of roots taken randomly from the soils. Bars followed by the same letter are not significantly different (P=0.05). Mean comparison was analyzed by using a two sample t-test.

Figure 7. Population densities of (A) total culturable heterotrophic aerobic bacteria and (B) DAPG-producing *P. fluorescens* strains F113 and L5.1-96 in the rhizosphere of wheat grown in transferred soils (original soil + steamed pasteurized Quincy virgin soil, 1:9 w/w ratio). Soils were first stored for a period of one year, cycled for five 3-week cycles, and then stored for a second time for 5 months. Values are the means of six roots from plants randomly selected. Bars within a cycle followed by the same letter are not significantly different (*P*=0.05) according to the two sample t-test.

Figure 8. Population dynamics of DAPG-producing *P. fluorescens* strains F113 (genotype K) and L5.1-96 (genotype D) in the rhizosphere of wheat. Cycle 10 is the population density on roots of wheat in the 10^{th} cycle of growth reported by Landa et al. (21) immediately prior to the soil being placed in bags for storage. During each cycle, wheat seedlings were allowed to grow for 3 weeks. Arrows indicate periods of storage; x= soils stored at room temperature for one year; y = soils stored at room temperature for 5 months, and z= soils stored at room temperature for 8 months. Values are the means of six roots and adhering soil from plants randomly selected. Mean values and standard deviations are presented.

Figure 1.

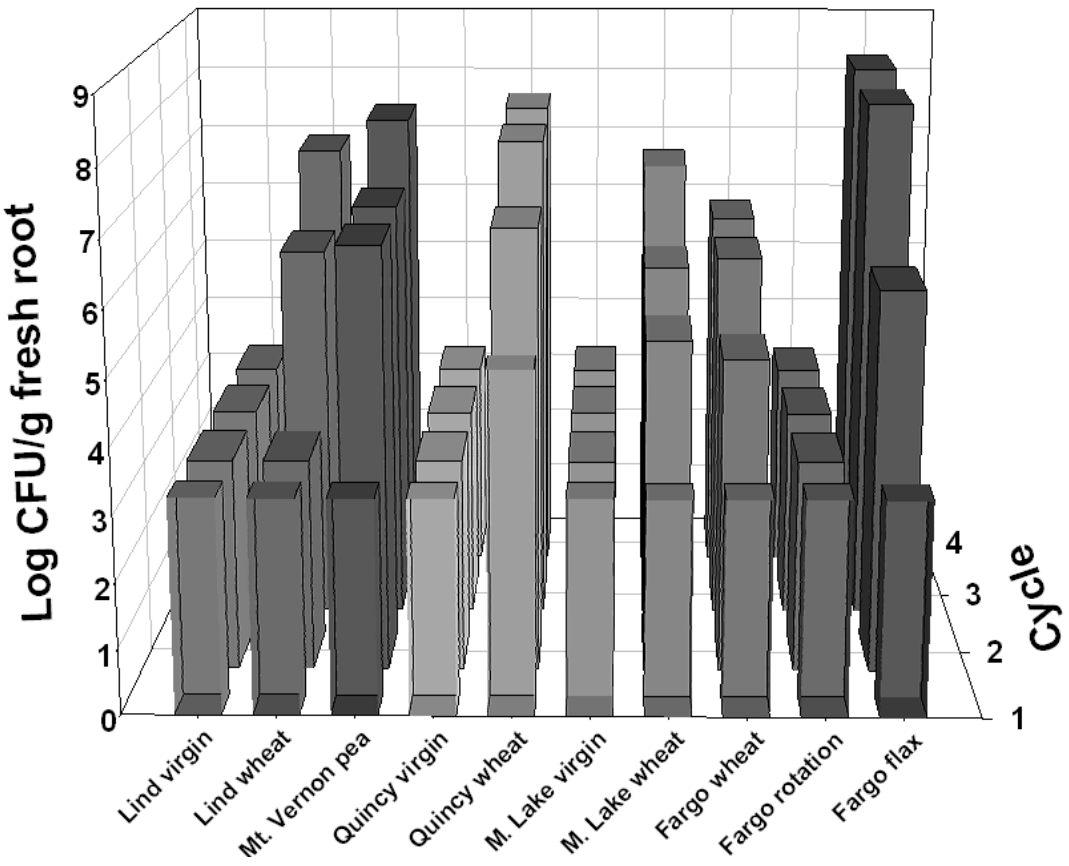

Log CFU/g fresh root
0
1
2
3
4
5
6
7
8
9
Cycle
1
2
3
4
Lind virgin
Lind wheat
Mt. Vernon pea
Quincy virgin
Quincy wheat
M. Lake virgin
M. Lake wheat
Fargo wheat
Fargo rotation
Fargo flax

Figure 2

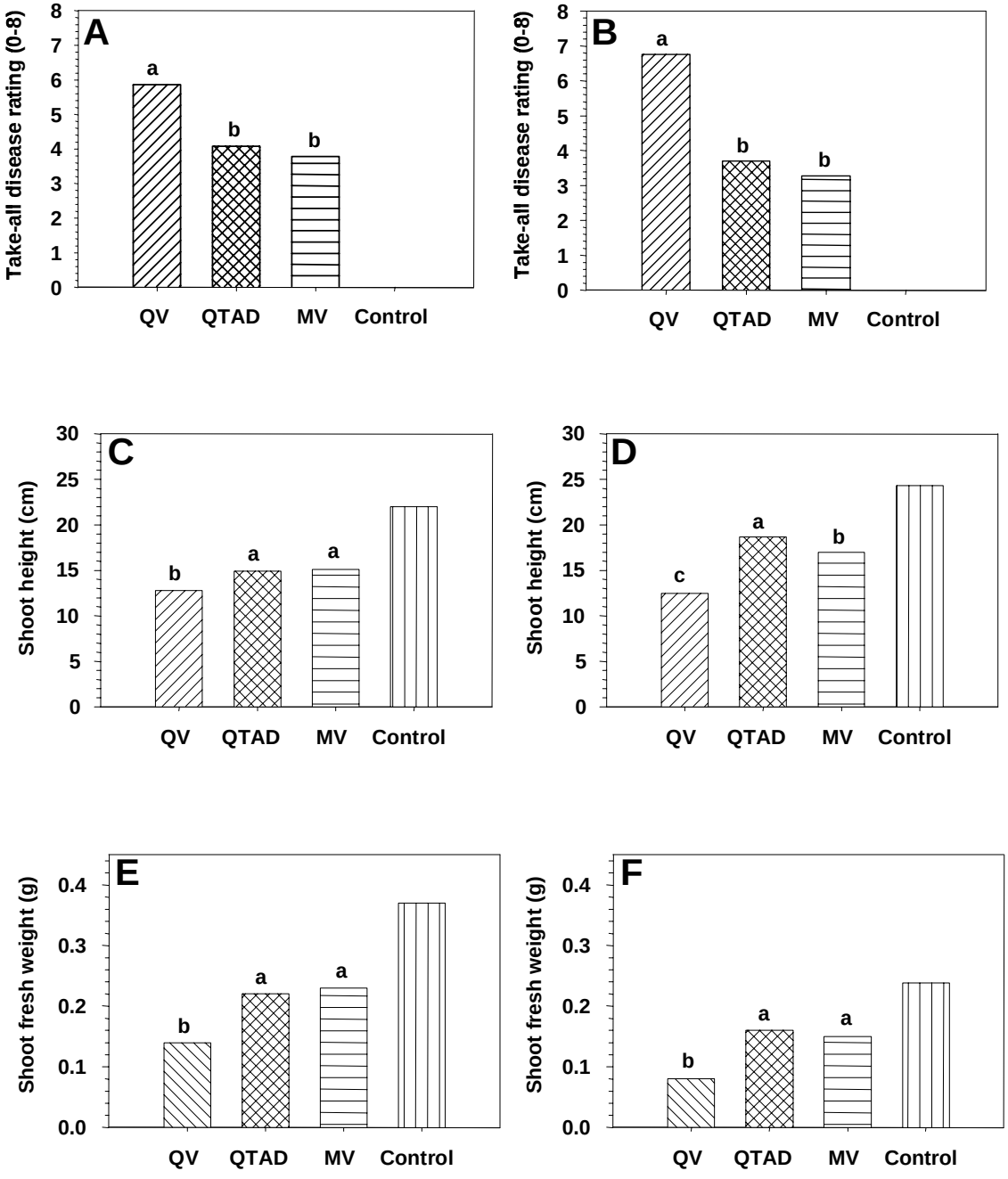

A
Take-all disease rating (0-8)
0
1
2
3
4
5
6
7
8
a
b
b
QV
QTAD
MV
Control
B
Take-all disease rating (0-8)
a
b
b
QV
QTAD
MV
Control
C
Shoot height (cm)
0
5
10
15
20
25
30
b
a
a
QV
QTAD
MV
Control
D
Shoot height (cm)
c
a
b
QV
QTAD
MV
Control
E
Shoot fresh weight (g)
0.0
0.1
0.2
0.3
0.4
b
a
a
QV
QTAD
MV
Control
F
Shoot fresh weight (g)
b
a
a
QV
QTAD
MV
Control

Figure 3.

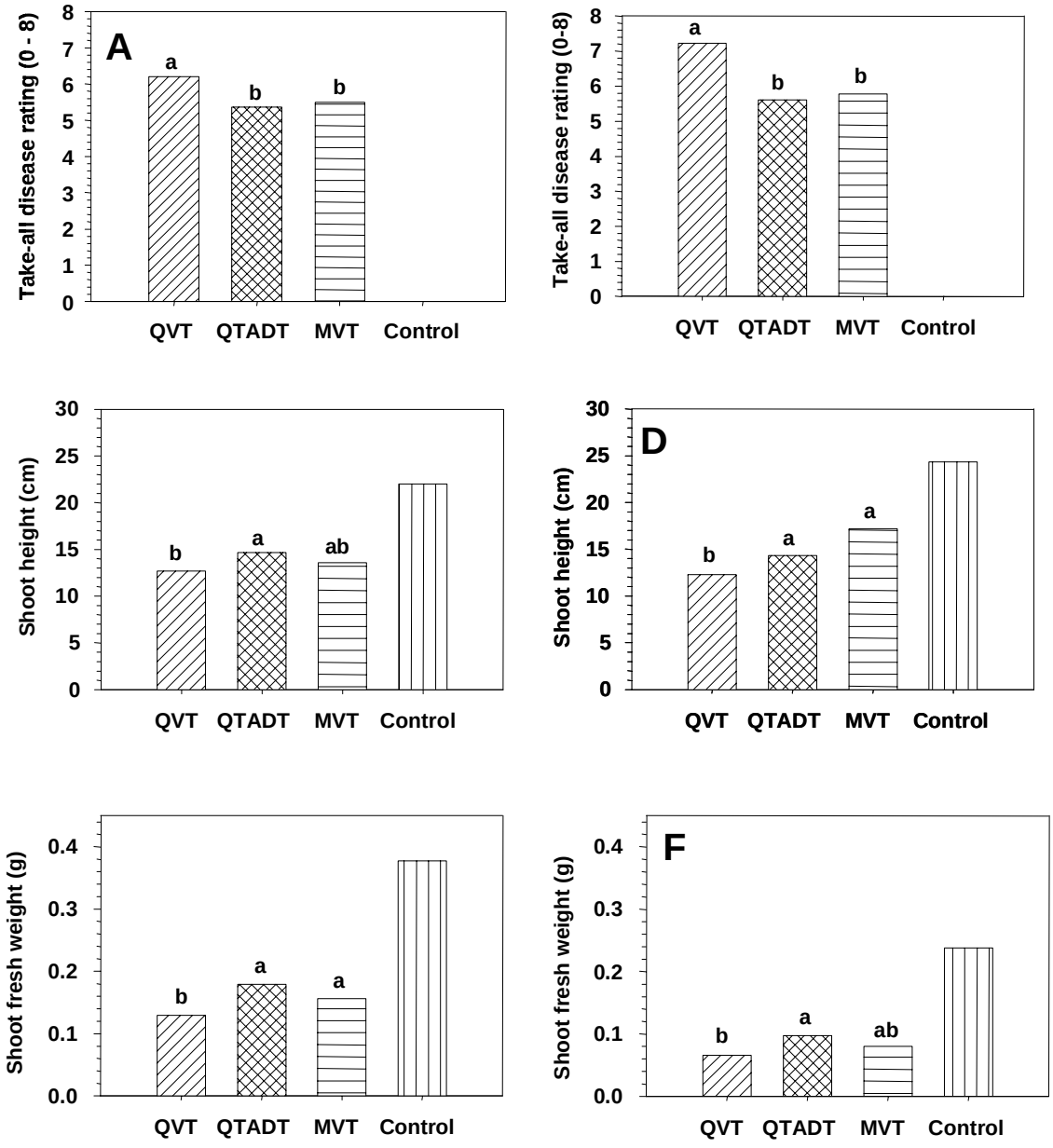

A
Take-all disease rating (0 - 8)
a
b
b
QVT
QTADT
MVT
Control
Take-all disease rating (0-8)
a
b
b
Shoot height (cm)
b
a
ab
D
a
Shoot fresh weight (g)
F
ab

Figure 4.

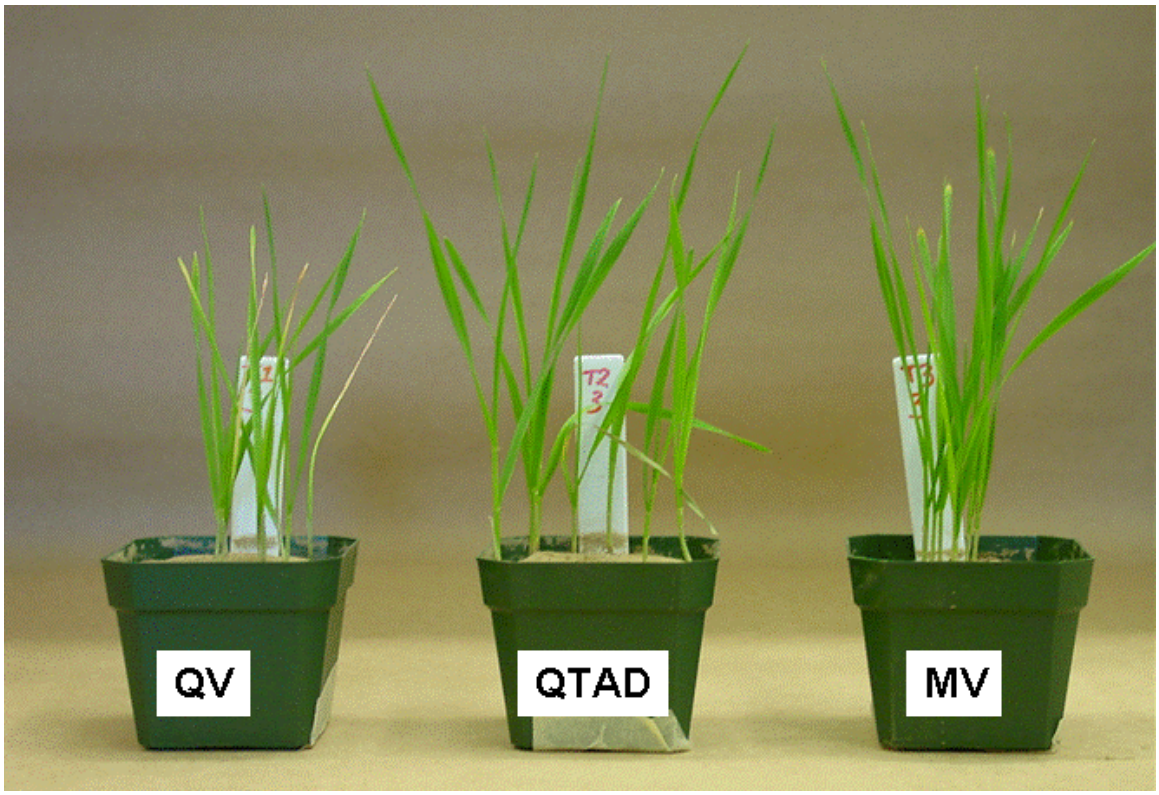


Figure 5.

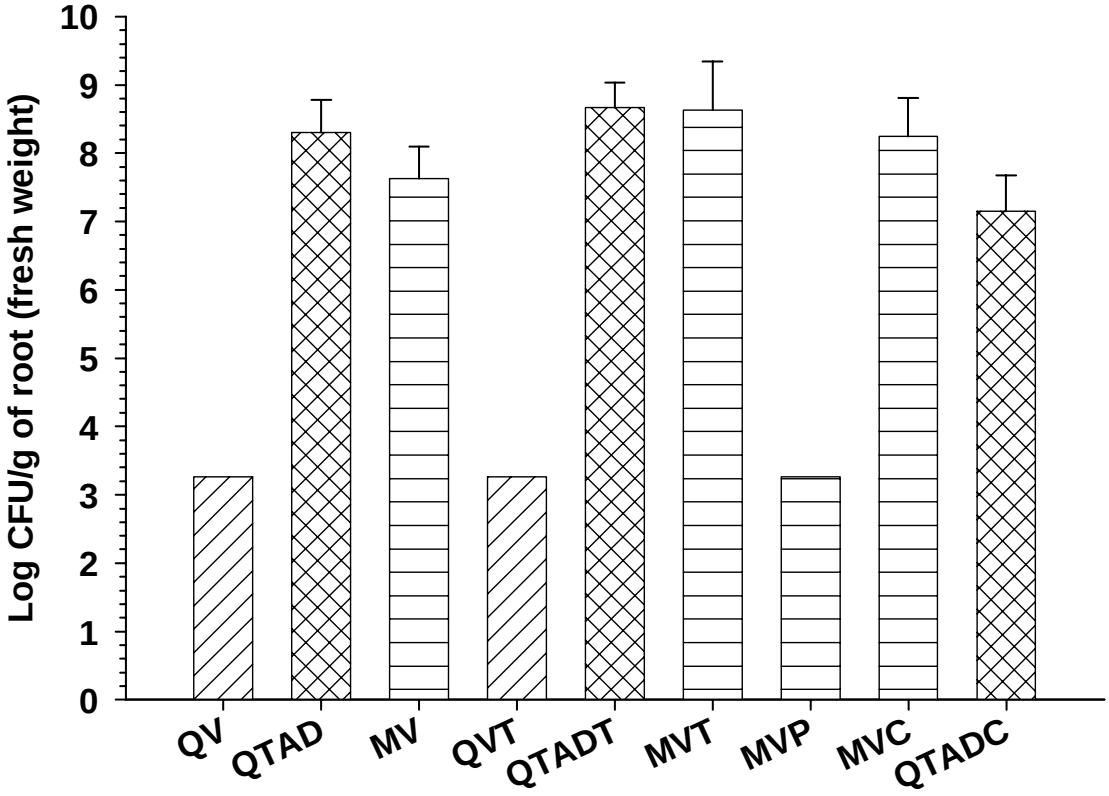

Log CFU/g of root (fresh weight)
10
9
8
7
6
5
4
3
2
1
0
QV
QTAD
MV
QVT
QTADT
MVT
MVP
MVC
QTADC

Figure 6

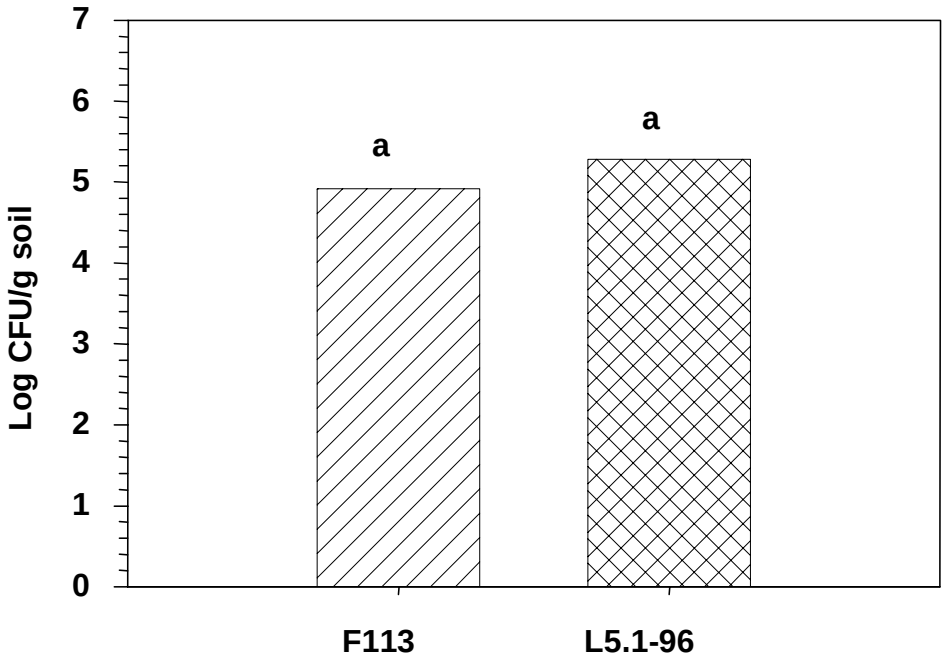


Figure 7

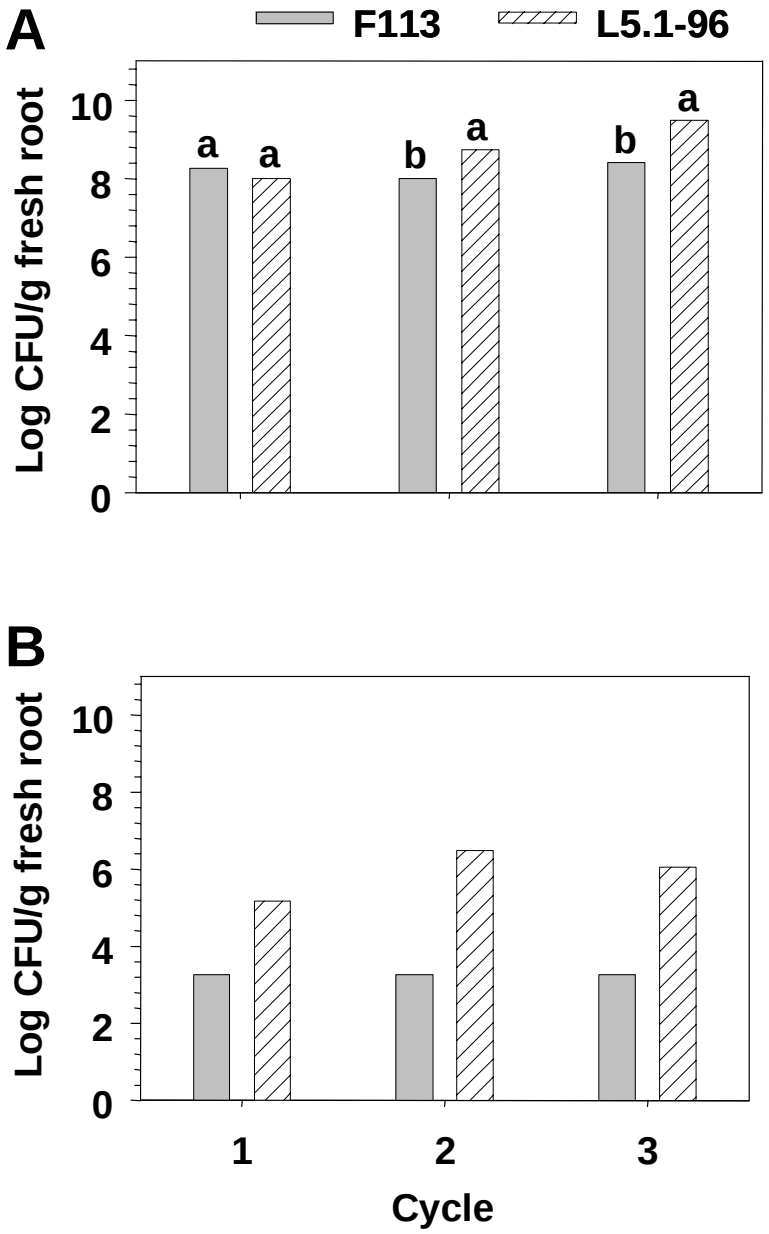
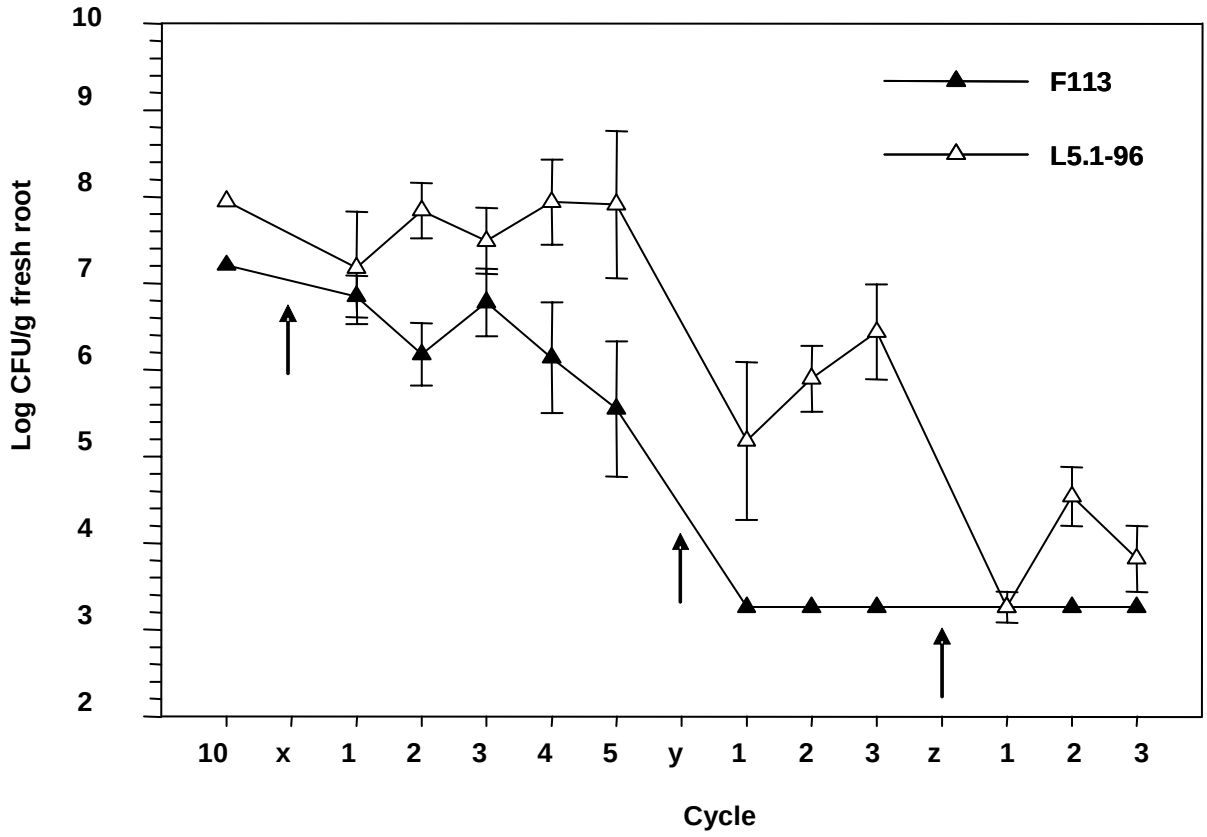

A
F113
L5.1-96
Log CFU/g fresh root
0
2
4
6
8
10
a
a
b
a
b
a
B
Log CFU/g fresh root
0
2
4
6
8
10
1
2
3
Cycle

Figure 8.


Log CFU/g fresh root
10
9
8
7
6
5
4
3
2
F113
L5.1-96
10
x
1
2
3
4
5
y
1
2
3
z
1
2
3
Cycle

CHAPTER 2

Lack of Evidence of Control of Pythium Root Rot of Wheat by Indigenous 2,4-Diacetylphloroglucinol-Producing *Pseudomonas fluorescens* from Take-all Suppressive Soils in Washington State.

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Keywords: non-target organisms, rhizosphere, Oomycetes.

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ABSTRACT

Rhizosphere populations of 2,4-diacetylphloroglucinol (DAPG)–producing *Pseudomonas fluorescens* enriched through wheat monoculture are a key component of take-all suppression in take-all decline soils from Washington State. In this study, to further evaluate the breadth of disease suppressiveness, two take-all decline soils from Washington State were evaluated for their suppressiveness against Pythium root rot of wheat, because *Pythium* spp. and *Gaeumannomyces graminis* var. *tritici* commonly co-inhabit wheat roots. Pythium root rot was evaluated in wheat seedlings grown in both take-all suppressive and conducive soils. Soils were artificially infested individually with isolates of four different *Pythium* spp. and then wheat was grown. Population sizes of DAPG-producing fluorescent pseudomonads in the rhizosphere, and plant and root measurements were determined. Potential suppressiveness was further investigated by transferring 10% (w/w) of the take-all suppressive and conducive soils to a common pasteurized soil infested by the *Pythium* spp. Take-all decline soils were not effective in reducing the symptoms associated with Pythium root rot when the soils were artificially inoculated individually with *P. abappresorium*, *P. irregulare* group I, *P. irregulare* group IV or *P. ultimum.*

In plants grown in infested conducive and suppressive soils, plant size and length of first leave, root tips, root lengths and root diameter were similar. Although no suppression of Pythium root rot was observed in wheat grown in the take-all decline soils, large population sizes of DAPG-producing *P. fluorescens* above the threshold required for disease suppression (10^5 CFU/g of root) were detected in the rhizosphere of

wheat grown in both infested and non-infested soils. Furthermore, *in vitro* assays showed that mycelial growth of *Pythium* spp. was inhibited by both DAPG-producing *P. fluorescens* strains and synthetic DAPG. However, minimal concentrations of DAPG required to completely inhibit mycelial growth of *Pythium* spp. were 5 to 10 times higher than those required to inhibit the take-all pathogen (3 μg/ml), possibly accounting for the lack of suppression. In conclusion, results obtained under our experimental conditions with wheat seedlings indicates the inability of indigenous DAPG-producing *P. fluorescens* in take-all decline soils from the Pacific Northwest to control root rots caused by the *Pythium* spp.

INTRODUCTION

Pythium spp. are important plant pathogens that inhabit agricultural soils throughout the world and cause pre-emergence and post-emergence damping-off and root rots in a great number of ornamentals and crop plants. Pathogenicity of *Pythium* spp. relies on their strong ability to rapidly colonize fresh plant residues (7). *Pythium* spp. are able to infect the germinating wheat seeds within 24 to 48 h after planting. Infection of the seedling can prevent germination of the seed (damping-off) or emergence of the seedling. If germination occurs the seedlings are stunted and deformed. The pathogen also invades and destroys root hairs and tips which limits nutrient and water uptake, ultimately reducing yield (5).

Soils suppressive to soilborne plant pathogens are the result of either biotic or abiotic factors that reduce disease even when the pathogen and susceptible host are present, and environmental conditions are suitable for disease (60). Most studies have focused on biologically based suppressiveness. Two types of disease suppressiveness are recognized: long-standing and induced. Long-standing suppressiveness is a natural characteristic of the soil, is independent of crop history, and its origin is not known. In contrast, induced suppressiveness results from crop monoculture, growing crops susceptible to the pathogen, or adding inoculum of the pathogen into the soil (60). Soils suppressive to *Fusarium oxysporum* (30), *Gaeumannomyces graminis* var. *tritici* (20), *Rhizoctonia solani* (62), *Thielaviopsis basicola* (56) and *Pythium* spp. (23, 31) are examples of those that have been described. In many suppressive soils, bacteria and fungi have been identified as antagonists to the plant pathogens. Mechanisms involved in the

suppression have been reported as competition for nutrients or infection sites, antibiosis, parasitism and induced systemic resistance (17, 60).

In Washington State, wheat fields from Quincy and Lind, WA, which had undergone monoculture for 22 and 28 years, respectively, are suppressive to take-all and have been designated as take-all decline (TAD) soils (38, 46, 48, 49). TAD is the spontaneous remission in the severity of take-all during continuous monoculture of wheat following an outbreak of the disease (60). Thus, TAD is a field phenomenon that requires the monoculture of a susceptible host, the presence of *Gaeumannomyces graminis* var. *tritici* (the causal agent of take-all), and at least one severe outbreak of the disease (60). Several antagonistic microorganisms have been implicated in the control of take-all (3, 4, 10, 25); however, in Washington State (USA) and in The Netherlands the build-up of populations of *Pseudomonas fluorescens* producing the antibiotic 2,4-diacetylphloroglucinol (DAPG) through continuous monoculture of wheat is responsible for take-all decline (55, 60).

Within a worldwide collection, DAPG-producing *P. fluorescens* are divided into 22 different genomic groups (genotypes A to T, PfY and PfZ) (15) on the basis of whole cell repetitive sequence based polymerase chain reaction (rep-PCR) by using the BOXA1R primer and restriction fragment length polymorphism (RFLP) and phylogenetic analysis of *phlD*, a key gene involved in the synthesis of DAPG (15, 28, 29, 34, 35, 36, 38). The biological significance of this classification was described by Landa *et al.* (27) and Raaijmakers and Weller (47), whose studies demonstrated that the genotype of a strain was predictive of its ability to colonize the roots of wheat and pea.

De la Fuente et al. (14) further showed the effect that the host crop genotype plays in the interaction with specific DAPG genotypes in the rhizosphere.

DAPG-producing *P. fluorescens* of different genotypes have been reported as biocontrol agents of Pythium diseases. These include Pythium diseases of cotton (21), cucumber (53), sugar beet (11, 13, 16, 50), soybean (36), pea (41), and tobacco (24).

Suppressive soils usually are known to be effective against a specific pathogen or related group of pathogens (60), but in most cases the true breadth of activity of a suppressive soil against other pathogens rarely has been explored. Given that *Pythium* spp. are ubiquitous in Pacific Northwest wheat fields, co-inhabit roots with *G. graminis* var. *tritici*, and are sensitive to biocontrol by DAPG producers, we hypothesized that Pythium root rot would be suppressed in TAD soils. In our study, two take-all decline soils in which indigenous DAPG producers are present and take-all suppression has been previously demonstrated (48) were selected to test for suppressiveness of Pythium root rot. The specific objectives were to: i) evaluate Pythium suppressiveness on wheat seedlings in these soils, ii) assess the effect of *Pythium* spp. infections on population sizes of DAPG-producing *P. fluorescens* in the rhizosphere of wheat, and iii) determine sensitivity of *Pythium* spp. isolated from Washington State wheat fields to DAPG and DAPG-producers.

We show that despite the ability of indigenous DAPG producers in TAD soils to establish very high population sizes on *Pythium* infected wheat roots, no natural suppression of root rot occurs. A variety of reasons for this lack of suppression are explored and discussed.

MATERIALS AND METHODS

Bacterial strains and growth media. *P. fluorescens* strains used in this study produce DAPG and are described in Table 1. All bacterial strains were cultured at 28°C in King's medium B (26). Stock cultures were stored in 1/3x KMB broth plus 35% glycerol at -80°C. Strains of *Pythium* spp. were routinely cultured on 1/5x homemade potato dextrose agar (PDA; potatoes 40 g, dextrose 4 g, agar 18 g, water 1 L) and stored on PDA and water agar (WA; agar 18 g, water 1 L) slants at 4°C.

Strains of DAPG-producing *P. fluorescens* were quantified in a modified fluorescent pseudomonad semi-selective medium, which consisted of 1/3x KMB broth (26) supplemented with ampicilin (40 μg/ml), chloramphenicol (13 μg/ml), and cycloheximide (100 μg/ml) (1/3x KMB $^{+++}$). The density of total culturable heterotrophic bacteria was quantified in 1/10x tryptic soy broth (TSB, Difco Laboratories, 3 g per liter) (1/10x TSB) supplemented with cycloheximide (100 μg/ml) (1/10x TSB^{+}) (28).

Pythium inoculum. Inoculum of *Pythium abappresorium*, *P. irregulare* group I, *P. irregulare* group IV, and *P. ultimum* was produced in mason jars containing a Ritzville silt loam agricultural soil collected from the Washington State University Dryland Experiment Station in Lind, WA. A 0.5-cm hole was drilled in the lids of the mason jars, and a 70-mm diam. filter disk (Fungi Perfecti, Corvallis, OR) was placed inside the lid to maintain air exchange and sterility. Soils were amended with 1% ground rolled oats, added to the jars and then autoclaved twice (18). *Pythium* spp. were transferred to the jar in the form of PDA plugs (half of a Petri plate 80 x 15 mm cut in 1-cm square). Sterile distilled water (50 ml) was added to the jars 48 h before the PDA plugs were added. The

jars were shaken and incubated for 3 weeks at room temperature (approximately 25°C), under the light. Prior to using the inoculum, propagule density in the mason jars was assessed by dilution plating on *Pythium* selective medium (40).

Soils. Take-all decline (TAD) soils, collected from fields near Quincy and Lind, WA (designated QTAD and LTAD, respectively) and previously showed to harbor populations of DAPG producers (50), were used in this study. Conducive soils from Quincy and Lind (designated as QV and LV, respectively) were collected from undisturbed sites that are naturally covered with native vegetation, and near the TAD fields. All soils were collected from the upper 30 cm of the soil profile, air dried, stored in plastic or metal buckets outside, and sieved through a 0.5 cm-mesh before used. Characteristics of these soils are described in Table 2.

Wheat cycling to activate indigenous DAPG-producing *P. fluorescens*.

All soils were cropped to spring wheat (*Triticum aestivum* cv. Penawawa) for at least three successive cycles of 3 weeks as described by Landa et al. (28) in order to "activate" indigenous microbial populations. At the end of each 3-week cycle of growth, six plants from each soil were randomly selected and roots with adhering soil were assayed to determine the population density of DAPG producers as described below. The soil was mixed, returned to pots and again sown to wheat, beginning the next cycle.

Suppression of Pythium root rot. In order to determine whether soils suppressive to take-all are also suppressive to Pythium root rot, inoculum of isolates of four *Pythium* spp. (*P. abappressorium*, *P. irregulare* group I, *P. irregulare* group IV or *P. ultimum*) were added to the take-all suppressive (QTAD and LTAD) and conducive (QV and LV) soils at a rate of 10^3 CFU/g soil and then 0.5% ground rolled oats were

amended to serve as a food base for the *Pythium* inoculum (19). The experiment was arranged in a completely randomized design with each treatment replicated 6 times, and a single pot serving as a replicate. Prior to sowing, soils were maintained wet, by adding 40 ml of tap water the first and the fourth day, for 7 days in a growth chamber at 15°C to stimulate the growth and establishment of the introduced *Pythium* spp. in the soils. Eight two-day-old pre-germinated wheat seeds (cv. Penawawa) were then sown in each pot containing 200 g of soil. Each pot received 40 ml of water after sowing. The wheat plants were incubated in a growth chamber at 15°C with a 12 h photoperiod for a total of 14 days. Pots were covered with a plastic film for 72 h after sowing to maintain humidity and to prevent the soil from drying before seedling emergence. After the plastic was removed, pots were watered at 3-day intervals and twice a week received 40 ml of a fertilizer solution 15-30-15 (Scotts Miracle-Gro Products, Port Washington, NY) until harvest. In some soils 40 ml of water containing metalaxyl (40 mg/ml) was added to suppress indigenous *Pythium* spp. and to test for the effect of *Pythium* infections on the population sizes of the DAPG produces in the rhizosphere.

Transfer of suppressiveness. The transferability of disease suppressiveness was tested essentially as described by Raaijmakers and Weller (48), by diluting each test soil into a steamed pasteurized Quincy virgin soil (30 min 60°C) (1:9 w/w). After the soils were mixed, inoculum of *Pythium* spp. was added, the soils were mixed again and dispersed among the pots. Pots were maintained wet during a 7-day period in a growth chamber as described above and then eight two-day-old pre-germinated wheat seeds (cv. Penawawa) were sown.

Plant and root measurements. At the end of each 2-week experiment, 2 plants per pot were randomly selected for root measurements. Roots of the plants were washed free of soil and debris using a stream of tap water and then were digitally scanned using a Hewitt-Packard Scanjet 5370C scanner and saved as TIF files. Root scans were analyzed using WinRhizo software (Regent Instruments Inc. Quebec, Canada). This software calculates total root length, average root diameter, and number of root tips. Measurement of these parameters is suitable to assess the effect of the Pythium infections on the growth of wheat roots (19). Shoot height and length of the first true leaf were determined on six plants randomly selected from each one of the replicates at the end of the 2-week experiment.

Determination of population densities of DAPG producers and total bacteria in the wheat rhizosphere. At the end of each experiment, six plants were randomly selected to determine the population sizes of bacteria in the rhizosphere. Root systems were removed from soil and carefully shaken to remove loosely adhering soil, leaving only tightly adhering rhizosphere soil. Roots were excised from the plants and placed individually into 50 ml screw-cap centrifuge tubes containing 10 ml of sterile distilled water. The tubes were shaken for 1 min on a Vortex mixer, and sonicated in an ultrasonic cleaner (Bransonic 521, Branson, Shelton, CT) for one minute, essentially described by Landa et al. (28). Population densities of DAPG producers were estimated using the PCR-based dilution-endpoint assay (28, 37). An aliquot (100 µl) of the root wash was serially diluted (1:3) in a 96-well microtiter plate pre-filled with 200 µl of sterile distilled water per well, and an aliquot (50 µl) of each dilution series was transferred to a 96-well plate containing fresh 1/3x KMB^{+++} broth, which is semi-selective for fluorescent

pseudomonads. Microtiter plates with 1/3x KMB^{+++} were incubated at room temperature (approximately 25°C) in the dark and bacteria in each well was assessed spectrophotometrically (Dynatech MR5000, Dynatech Laboratories, Burlington, MA) after 96 ± 4 h. An absorbance at 600 nm (OD_{600}) of ≥ 0.07 was scored as positive for growth (37). The terminal dilution culture (TDC) is the greatest dilution (well) showing bacterial growth. Aliquots from wells showing growth were tested for the presence of DAPG producers (*phl*D^+ *Pseudomonas* spp.) by PCR analysis as described below, and population densities were determined on the basis of the last well where the *phl*D signal was detected.

The population density of total culturable aerobic heterotrophic bacteria in each sample was determined by transferring aliquots (50 µl) from the serially diluted (1:3) root washings into a 96-well microtiter plate containing 1/10x TSB^+. Microtiter plates were incubated at room temperature in the dark, and bacterial growth was assessed spectrophotometrically as described above, after 48 ± 4 h. The terminal dilution in the microplate showing positive growth (OD_{600} ≥ 0.07) was used to calculate the total population of culturable bacteria in a sample.

PCR amplification and detection of *phlD*. PCR amplifications to detect *phlD* were conducted on aliquots of cultures from the microtiter plates described above or from pure bacterial cultures after freezing at -80°C for a minimum of one hour and essentially as described previously (28, 37). Briefly, amplification was performed in a 25 µl reaction volume containing 2.5 µl of thawed whole cell template and the generic primers B2BF and BPR4 (37), using a PTC-200 thermal cycler (MJ Research, Inc., Watertown, MA). The resulting PCR products were separated in an 1.5% agarose gel in 0.5x tris-borate-

EDTA (TBE) buffer at 125 V for 2 h. Banding patterns were visualized by ethidium bromide staining and scored by comparison to a 1 Kb bp ladder.

In vitro assay for inhibition of mycelial growth by DAPG-producing strains. Sensitivity of *Pythium* spp. to DAPG-producing *P. fluorescens* strains belonging to eight different DAPG genotypes was tested by an in vitro assay based on inhibition of mycelial growth. The assays were conducted on homemade PDA, 1/5x homemade PDA and KMB. The pH of the media was adjusted to 7.0 before autoclaving. Aliquots (3 µl) of each bacterial strain (OD_{600} nm = 0.1 aprox. 3 x 10^5 CFU) were placed at equidistant spots (3 cm) from the center of the plate. Sterile distilled water (SDW) was spotted as a control. Agar plugs (4 mm in diameter) of each of the ten *Pythium* spp. actively growing in 1/5x PDA were placed in the center of the plate 48 h later. Plates were incubated at room temperature in the dark. The assay was ended when fungal colonies in the treatment control reached the spot were SDW was spotted.

A modification of the inhibition index (I): I = IN – T described by Ownley et al., (43) was used to determine the effect of the bacterial strains on inhibition of mycelial growth of the *Pythium* spp. Briefly, the inhibition index is obtained by measuring the inhibition zone (IN) distance from the edge of the bacterial colony to the growing edge of the fungus and divided by the total distance (T) from the edge of the agar plug to the edge of the bacterial colony. Resulting data are between index 0 and 1 meaning amount of inhibition compared with a control.

Inhibition of *Pythium* spp. by synthetic DAPG. The effect of synthetic DAPG (Toronto Research Chemicals Inc., North York, Ontario, CANADA) on *Pythium* spp. was tested on PDA. DAPG was dissolved in methanol. Agar discs (4 mm diameter) from

mycelia actively growing on 1/5x PDA were placed in 24-well microtiter plates (1.1 cm diameter) previously filled with PDA (0.8 % agar, pH adjusted to 7 before autoclaving) amended with DAPG at 0, 5, 10, 15, 20, 25, 30, 35, 40 µg/ml concentrations. In the treatment control no DAPG was added and an additional control was PDA amended with the highest methanol concentration used in the treatments. Microtiter plates were incubated at room temperature (25 °C) in the dark. Inhibition of mycelial growth was evaluated when the mycelial growth in the control treatments reached the entire surface of the well. Each treatment consisted of 6 repetitions (1 well = 1 repetition) and the experiments were repeated 2 times.

Statistical analysis. Data were analyzed by using the Statistix program (version 8, Analytical Software, Tallahassee, FL). Treatments in the disease suppression assays were arranged in the growth chamber as a completely randomized design. One-way analysis of variance was conducted to test for differences between treatments. Bacterial populations data were log transformed before statistical analysis. Significant differences between means were determined using Fisher's protected least significant difference. Bioassay experiments were arranged in a randomized complete design and data were rank transformed before statistical analysis. When no transformation was available, significant differences between means were determined by the Kruskal-Wallis test.

RESULTS

Indigenous populations of DAPG-producing *P. fluorescens* in take-all decline soils. The process of 3-week growth cycles, used to activate the indigenous microflora

and populations of DAPG producers resulted in population sizes above log 5 CFU/g of fresh root of DAPG producers in the rhizosphere of wheat growing in the Quincy and Lind TAD soils. In contrast, in the rhizosphere of Quincy and Lind virgin take-all conducive soils, densities of DAPG producers were below the detection level (data not shown). Once the soils harbored population sizes above log 5 CFU/g of fresh root, the Pythium root rot suppression assays were conducted.

Suppression of Pythium root rot in TAD soils: plant height and length of first true leaf. In order to test whether QTAD and LTAD soils suppressive to take-all were also suppressive to Pythium root rot of wheat, four different isolates belonging to different *Pythium* spp. (*P. abappressorium*, *P. irregulare* group I, *P. irregulare* group IV or *P. ultimum*) were artificially introduced in the soils and then Pythium root rot was measured. The transferability of suppression was also evaluated by adding the *Pythium* spp. into the take-all suppressive and conducive soils that were diluted (1:9 w/w) with a pasteurized Quincy virgin soil.

In eight independent experiments involving each one of the *Pythium* spp. tested and both TAD soils, Pythium root rot caused variation in all of the parameters evaluated including plant height, length of the first true leaf, total root length, number of root tips and average of root diameter.

All four *Pythium* spp. introduced into the take-all suppressive and conducive soils from Quincy and Lind, WA caused a reduction in plant height of wheat plants in all four soils as compared to non-inoculated soils (Figs. 1, 3). In general, Pythium root rot was more severe in plants growing in Quincy soils than in Lind soils based on the height measurements. Root infections of *Pythium* spp. resulted in an average reduction in shoot

height of 28% in plants grown on Quincy soils and an 18% reduction of shoot height in plants grown in Lind soils, when compared with plants grown in non-infested soils. Measurement of the length of the first true leaf also showed reductions of plant growth in plants growing in soils infested by *Pythium* spp. (Figs. 2, 4).

In suppressive and conducive soils from Quincy, WA *P. irregulare* group I and *P. irregulare* group IV caused the greatest reduction of shoot height and length of the first true leaf in the wheat plants. No significant difference in shoot height was observed between plants growing in the suppressive and conducive soils from Quincy when *P. abappressorium* (P=0.48) or *P. irregulare* group I (P=0.22) were present. When *P. irregulare* group IV (P= 0.049) or *P. ultimum* (P=0.001) were present in the soils, differences occurred between plants grown in the natural undiluted soil and soil transfer treatments (Fig.1); however, no significant differences were observed between plants grown in the conducive and suppressive soils (analysis not shown). On the basis of measurements of length of first true leaf, and with the exception of *P. irregulare* group I, all *Pythium* spp. caused significantly greater reductions in the length of the first true leaf on plants grown in the suppressive soil as compared to the conducive soil in the experiments with undiluted natural and transferred soil treatments (Fig. 2).

In the experiments conducted using soils from Lind, WA, no significant difference in shoot height was observed between plants grown in soils infested by *P. abappressorium* (P= 0.76) or *P. irregulare* group IV (P=0.39) (Fig. 3). However, when *P. irregulare* group I (P=0.01) or *P. ultimum* (P= 0.008) were infesting the soils, significant difference was observed between the conducive and suppressive natural undiluted soils with LTAD resulting in taller plants (Fig. 3). However, no differences

were observed in the soil transfer treatments (analysis not shown). *Pythium* spp. caused a reduction of the length of the first true leaf in comparison with the control treatment (Fig. 4). No significant differences in length of the first true leaf were observed between plants growing in suppressive or conducive soils infested by the *Pythium* spp.

Suppression of Pythium root rot in TAD soils: total root length, number of root tips and average diameter of root. Measurements of root scans analyzed by using the WinRhizo software, including total root length, average root diameter and number of root tips, are shown in Figures 5-10. All *Pythium* spp. caused a reduction of total root length and number of root tips of wheat seedlings grown in Quincy and Lind soils in comparison with a control treatment (no inoculum added). In comparison with the control treatment, almost all treatments showed an increase of average of root diameter, apparently due to the destruction of root tips, root hairs and the reduction of the root length caused by *Pythium* spp.

In the Quincy soils, with the exception of *P. irregulare* group IV *(P*=0.001) and *P. ultimum* (*P*=0.001), no differences in total root length were observed between plants growing in suppressive and conducive soils infested with *P. abappressorium* (P=0.360) and *P. irregulare* group I (*P*=0.528). In measurements of the number of root tips, no significant differences were observed on roots growing in the Quincy soils infested by *P. abappressorium* (*P*=0.570). Significant differences were observed in the number of root tips of wheat grown in soil infested by *P. irregulare* group I (*P*= 0.001)*, P. irregulare* group IV (*P*= 0.001)*, and P. ultimum* (*P*=0.003). Greater numbers of root tips were observed in roots of wheat grown in the conducive soils in both natural and transferred treatments when *P. ultimum* was present (Fig. 6). With the exception of *P. ultimum*, all

Pythium spp. caused an apparent increase in root diameter in comparison with the control (Fig. 7).

In the experiments conducted using soils from Lind, WA, significant differences in total root length were only observed when *P. irregulare* group I (*P*=0.001) infested the soils: the total root length was larger in plants grown in the suppressive than in the conducive soils both in natural and transferred treatments (Fig. 8). In measurements of the number of root tips, significant differences were observed only when *P. abappressorium* (*P*=0.001) and *P.* irr*egulare* group I (*P*=0.001) were infesting the soils (Fig. 9). The effect of each one of the *Pythium* spp. caused an increased in root diameter in comparison with the uninfested control treatment (Fig. 10). Significant differences in root diameter among soil treatments were observed when *P. irregulare* group I (*P*=0.014), *P. irregulare* group IV (*P*=0.027), or *P. ultimum* (*P*=0.001) infested the soils.

Overall the experiments showed that soils suppressive to take-all were not able to consistently reduce the severity of Pythium root rot as compared with soils conducive to the disease during the seedling phase of wheat growth.

Total culturable bacteria and total *Pseudomonas* spp. in the rhizosphere of wheat. The population densities of culturable aerobic heterotrophic bacteria in the rhizosphere of wheat grown in the Quincy soils infested with *Pythium* spp. ranged between log 8.2 and 9.6 CFU/g of fresh weight of root. Only when *P. irregulare* group IV was present in the soil, significantly (*P*= 0.036) larger population densities of heterotrophic bacteria were observed in the rhizosphere of wheat grown in the suppressive soils as compared with the conducive soils. In the rhizosphere of wheat grown in Lind soils infested with *Pythium* spp., no significant differences (*P*=0.05) were

observed in population densities of total culturable aerobic heterotrophic bacteria (Table 3). Larger population sizes of total *Pseudomonas* spp. were observed in the Quincy suppressive soils as compared with the conducive soils only when *P. irregulare* group IV (P=0.036) or *P. irregulare* group IV (P= 0.019) were present. In contrast, significant (P=0.001) differences in total *Pseudomonas* spp. were only observed in the rhizosphere of wheat grown in Lind soils infested by *P. ultimum* (Table 3).

DAPG-producing *P. fluorescens* in the wheat rhizosphere. Population sizes of DAPG-producing *P. fluorescens* >log 5 CFU/g fresh weight of root were consistently recovered only from the Quincy TAD and Lind TAD soils (Fig. 11). DAPG-producing *P. fluorescens* were below the detection level in Quincy and Lind uncropped (virgin) soils. Population sizes of indigenous DAPG-producing *P. fluorescens* ranged between log 7.12 and 9.05 CFU/g of fresh weight of root in Quincy soil. When *P. abappressorium*, *P. irregulare* group IV or *P. ultimum* were present in the soil, population densities of DAPG producers were significantly (P=0.001) greater as compared to the control. In Lind soil, the population sizes ranged between log 8.63 and 9.36 CFU/g fresh weight of root. The presence of *P. abappressorium*, *P. irregulare* group IV or *P. ultimum* did not significantly (P=0.05) increase the densities of the DAPG producers as compared to the control. When *P. irregulare* group I was present in the soil, DAPG producers were significantly (P=0.001) greater in the LTADT treatment than in the control or the LTAD treatment (Fig, 11).

Effect of DAPG-producing *P. fluorescens* strains on mycelial growth of *Pythium* spp. In vitro assays revealed that strains of *P. fluorescens* belonging to different genotypes of DAPG producers demonstrated differential activity against *Pythium* spp.

Stronger inhibition of mycelia growth was observed on KMB (inhibition index average 0.28) than on 1/5x PDA (inhibition index average 0.19). On 1/5x PDA, strains Q2-87 (genotype B), F113 (genotype K) and phL1C2 (genotype M) caused greater inhibition of the *Pythium* spp. tested than strains Pf-5 (genotype A), Q8r1-96 (genotype D), 5MR2R (genotype E), 1M1R (genotype L) and MVW4 (genotype Q). On KMB, strain Pf-5 showed significant greater (P=0.001) inhibition of *P. irregulare* group I and *P. ultimum* as compared to the remaining strains, and was among the strains that showed the greatest reduction of *P. abappressorium* and *P. irregulare* group IV (Table 4). When data from PDA assay were pooled and analyzed (analysis not shown), *P. ultimum* was the least sensitive of the *Pythium* spp. Strains phL1C2, Q2-87, and F113 showed the greatest reduction on mycelial growth of *Pythium* spp. When pooled data from the KMB assay were analyzed, *P. irregulare* group I and *P. irregulare* group IV were the most sensitive to the effect of the DAPG producers. Strains Pf-5, Q8r1-96, F113 and Q2-87 caused the greatest reduction of mycelial growth on *Pythium* spp.

Effect of pure DAPG on mycelial growth of *Pythium* spp. The effect of the exposure of *Pythium* spp. to pure DAPG caused complete inhibition of mycelial growth of *Pythium* spp. at concentrations between 15 and 30 µg/ml. *P. heterothallicum* was the most sensitive species, whereas *P. abappresorium, P. irregulare* group I*, P. paroecandrum,* and *P. rostratifingens* were less sensitive to DAPG (Table 5). CE_{50} were obtained for each one of the *Pythium* spp., by correlating log concentrations of DAPG and percentage (transformed to probit) of mycelial inhibition (22). Analysis of CE_{50} showed that *P. paroecandrum*, *P. irregulare* group I and *P. ultimum* were the least sensitive species and were significantly different (P=0.001) from the rest of *Pythium* spp.

tested. *P. rostratifingens*, *P. heterothallicum,* and *P. attrantheridium* were among the species more sensitive to pure DAPG (Table 5).

DISCUSSION

The goal of this study was to evaluate whether the suppressiveness of take-all decline soils from Washington State is active against other soilborne pathogens. We focused our study on the ability of indigenous populations of DAPG producers in two TAD soils from Quincy and Lind, WA to suppress Pythium root rot. The TAD soils were compared to homologous conducive soils from nearby non-cropped sites with similar soil properties (Table 2), but which lacked populations of DAPG-producers above the threshold density required for disease suppression (49). Pythium root rot was selected as the target disease because *Pythium* spp. are ubiquitous in agricultural soils and always co-inhabit roots infected by the take-all pathogen; and DAPG-producing *P. fluorescens* are effective biocontrol agents against several soilborne pathogens including *Rhizoctonia solani* (21), *Fusarium oxysporum* f. sp. *radicis-lycopersici* (53), *Thielaviopsis basicola* (56)*, and Gaeumannomyces graminis* var. *tritici* (49) in geographically and chemically diverse soils. They also suppress diseases caused by *Pythium* spp. on cotton (21), cucumber (53), pea (41), and sugarbeet (13) as well as take-all of wheat (48, 55).

We first cycled the soils to wheat to increase the population density of indigenous DAPG-producing *P. fluorescens* to a level >log 5 CFU/g of fresh weight of root, the threshold level known to be required for disease suppression (49). Isolates of *P. abappressorium*, *P. irregulare* group I, *P. irregulare* group IV or *P. ultimum* were

introduced into the soils as the target pathogens of suppression, because they are among the *Pythium* spp. most virulent or widespread in wheat fields in WA State (18, 44). *Pythium* spp. were introduced into the soil at a density 10^3 CFU/g soil and ground rolled oats were added to the soil at 0.5 % to serve as a food base to increase inoculum potential. The rate of inoculum introduced into the soils is commonly found in field soils. Cook et al. (8) showed that 23% of 39 soils samples from wheat fields collected in eastern Washington and northern Idaho had between 700 and 1000 propagules of *Pythium* spp./g of soil.

Our disease assay and the rate of inoculum used in the experiments caused disease symptoms consistent with those seen in the field on wheat seedlings: reduction of shoot height, length of the first true leaf, and total root length, and the destruction of root tips. By using 2-day-old pre-germinated seeds, we avoided the problem of damping off, symptoms that are less commonly seen in the field.

A comparison of the severity of Pythium root rot on wheat grown in soils either suppressive or conducive to take-all or these soils diluted (1:9) with pasteurized soil indicated that the TAD soils from Quincy and Lind, WA were not suppressive to *P. abappressorium*, *P. irregulare* group I, *P. irregulare* group IV or *P. ultimum* on wheat during the seedling phase. In most cases, measurements of plant height, length of the first true leaf and root measurements (including root length, number of root tips and root diameter) from plants grown in the suppressive soils were similar to those of plants grown in the homologous conducive soils. In a few cases, when significant differences occurred on wheat in the suppressive and conducive soils, those differences were not consistent. For example, length of first true leaf was less on plants grown in the QTAD

soil than in the Quincy virgin soil (Fig. 2). In contrast, shoot height was less in plants grown in Lind conducive virgin soil infested by *P. irregulare* group I or *P. ultimum* as compared to wheat in the LTAD soil (Fig. 3). The lower disease severity observed in a few cases in plants grown in the conducive soils as compared to the suppressive soils can probably be attributed to the "general suppression" (60) that results from the effect of the general microbial population in the soil. However, general suppression is not able to be transferred into a pasteurized conducive soil, which explains the fact that sometimes in natural undiluted soils, plants were healthier in the conducive soils compared to the suppressive, but the disease severity was similar when soils were diluted (1:9 w/w) with pasteurized conducive soil (Fig. 3).

The absence of suppression of Pythium root rot on wheat grown in the TAD soils containing indigenous DAPG-producing *P. fluorescens* was at first surprising given that our *in vitro* assays showed that the mycelial growth of all *Pythium* spp. was inhibited to some extent by isolates of genotypes previously isolated from the TAD fields in Washington State (28, 38, 47). However, genotypes of DAPG-producers varied considerably in activity against the *Pythium* spp. For example, strains F113 (genotype K) and phL1C2 (genotype M), isolated from sugar beet (13) and tomato, respectively, showed the highest reduction of mycelia growth of *Pythium* spp., in both KMB and PDA. Both of these genotypes are known to occur only in European soils (15). Surprisingly, of the strains from Washington State soils (Q2-87, genotype B; Q8r1-96, genotype D; 5MR2R, genotype E; 1M1-96 Genotype L; and MVW4-2R, genotype Q), only strain Q2-87 was among the strains with highest reduction on mycelial growth on both KMB and PDA media. The other Washington State isolates showed significantly less inhibition

than the European strains (Table 4). However, isolates belonging to the B genotype are not the most abundant in the TAD soils (45) and previous studies have shown that isolates belonging to D genotype can outcompete B genotype isolates *in vitro* and in the rhizosphere of wheat when they are applied in combination (27, 57).

The absence of Pythium root rot suppression in the TAD soils may also be related to a lower sensitivity of *Pythium* spp. to DAPG as compared to *G. graminis* var. *tritici*. Our assays with synthetic DAPG showed that the minimal concentration of the antibiotic needed to inhibit mycelial growth of *Pythium* spp. isolated from Washington wheat fields ranged from 15 to 30 µg/ml, with most inhibited by 25-30 µg/ml. In contrast, *G. graminis* var. *tritici* is inhibited by only 3µg/ml. Thus, *Pythium* spp. are 5 to 10 times less sensitive to DAPG the take-all pathogen (Table 5).

Pythium spp. belong to the Kingdom Stramenopila and phylum Oomycota (1). Plant parasitic oomycetes are fungal-like organisms, characterized by the occurrence of a variety of stages in their life cycle: mycelia, zoosporangia, zoospores, encysted zoospores and oospores (1). These structures differ in importance as inoculum sources depending on the *Pythium* spp. and the crops attacked, and also differ in sensitivity to DAPG (54). The differential sensitivity of the various *Pythium* structures to DAPG is another factor that may explain the inability of TAD soils to suppress Pythium root rot. The inoculum used to infest the soils in our experiments included mostly mycelia in addition to oospores, sporangia and hyphal swellings, which were colonizing the ground oats added to the autoclaved soil (19). Mycelia were found to be the least sensitive stage of *Pythium ultimum* to the pure DAPG (54). Zoospores are the most sensitive stage in the life cycle of *Pythium ultimum* var. *sporangiferum*, however zoospores are only important in

cropping systems with water in abundance but they do not play an important role in the epidemiology of Pythium root rot of wheat in the Pacific Northwest. It is also noteworthy that in our studies, soils were artificially infested by individual *Pythium* spp., however in the field, between one and up to six *Pythium* spp. have been detected in individual soil samples collected from wheat fields in eastern Washington State (44, 51). This complexity of populations of *Pythium* spp. represents an additional factor for the DAPG producers to overcome in field conditions. For example, in the field, Milus and Rothrock (39) evaluated efficacy of bacterial treatments including DAPG producers for controlling Pythium root rot of winter wheat and they found that root rot suppression was inconsistent across experiments.

Another possibility for the lack of disease suppression in our experiments is that plant pathogens can affect the production of antibiotic in some biocontrol strains. Interactions between DAPG genotypes and plant pathogens can lead to differences in antibiotic production (9) and distinct DAPG genotypes can perform differently against oomycete pathogens (36). For example fusaric acid produced by *Fusarium oxysporum* f. sp. *radicis-lycopersici* completely abolished the DAPG biosynthesis in *P. fluorescens* CHA0 but not on *P. fluorescens* Q2-87 (9). However, in a similar experiment Notz et al. (42) found that root infection in cucumber and maize by *P. ultimum* stimulated the bacterial gene expression of *phlA* in *P. fluorescens* CHA0. Our studies did not consider DAPG production in the rhizosphere of wheat infected by *Pythium* spp. but it is a topic of current study.

Infection of roots by soilborne pathogens can affect both the population sizes of the total microflora (59) and indigenous and introduced biocontrol agents (4, 12, 33, 42).

For example, Mazzola and Cook (34) reported that rhizosphere populations of *P. fluorescens* strains 2-79 and Q72a-80 on wheat were the same as or higher on roots infected or not infected by the pathogens *R. solani* and *G. graminis* var. *tritici* from 20 to 40 days after inoculation. However, populations of the two bacteria declined more rapidly on roots infected by *P. irregulare*. Thus, differences in root colonization abilities of these bacteria were related to the type of lesion produced by the pathogens. In addition, Fedi et al. (12) found that *Pythium ultimum* adversely affected the ability of *P. fluorescens* strain F113 to colonize the sugar beet rhizosphere. In contrast, populations of *P. fluorescens* CHA0 in the rhizosphere were larger on cucumber but not in corn when *P. ultimum* was infecting the roots (9). Given the results of Mazzola et al. (33), initially we thought that *Pythium* infections would reduce rhizosphere colonization by the DAPG producers and thus their ability to protect the roots of wheat against *Pythium* spp. in the take-all suppressive soils. However, populations of the indigenous DAPG-producing *P. fluorescens* were not reduced in the rhizosphere of wheat grown in the TAD soils infested by *Pythium* spp. Aditionally, in the experiments of transferability of suppression, the rhizosphere of wheat grown in soils containing only 10% of the original TAD soils and 90% of a pasteurized conducive soil also harbored high populations sizes of DAPG producers in the presence and absence of *Pythium* spp. (Fig. 11). In LTAD soils, rhizosphere populations of DAPG producers were essentially the same on healthy and diseased plants and remained above the threshold density required for pathogen suppression. In contrast in QTAD soils, significantly larger populations of DAPG-producers (up to 100 fold greater) were detected on the rhizosphere of wheat infected by *P. abappressorium*, *P. irregulare* group IV or *P. ultimum*.

A final reason for the lack of Pythium suppression in the TAD soils may relate to differences in the epidemiology of take-all and Pythium root rot. *G. graminis* var. *tritici* emerges from colonized pieces of roots and crowns remaining in the soil from the previous crop. The fungus colonizes new roots as dark runner hyphae and then infection hyphae penetrate the roots throughout the season. The fungus spreads from seminal to crown roots and finally to the base of the tillers and up the tillers if conditions are suitable. In contrast, *Pythium* spp. attack the seed within 24 to 48 h after planting, rapidly establish inside the young roots, and destroys root hairs and tips. Indigenous populations of DAPG producers generally remain at low levels ($<10^4$ CFU/g of soil) in the bulk soil and are stimulated by the emergence of roots pushing through the soil. In the case of take-all, DAPG producers have a longer window of opportunity to establish threshold population sizes on the roots to inhibit infection as compared to Pythium root rot. *Pythium* infection is so fast that *Pythium* spp. become established in the roots before DAPG producers, which are distributed throughout the soil, can reach threshold populations sufficient to suppress *Pythium* infections. In contrast to indigenous DAPG producers, the ability of DAPG producers applied on seed or seed pieces to suppress Pythium diseases on a variety of crops (11, 13, 41, 50) is due to the placement of large doses of bacteria near infection courts, which can preempt infection.

In conclusion, our studies focused on elucidating the potential role of indigenous DAPG producers present in take-all decline soils in the suppression of *Pythium* spp. on wheat. To our knowledge, there has been no previous study of the suppressiveness of TAD soils to Pythium root rot. We utilized protocols which have been well-described for studying the suppressiveness of TAD soils against *G. graminis* var. *tritici* during the

seedling phase of wheat. They involved amending suppressive or conducive soils or soils diluted (1:9 w/w) with pasteurized soils and measuring symptoms of *Pythium* infection on wheat seedlings. We assumed that as with take-all suppressiveness, suppression identified during the seedling phase, would correlated with the observed in the field. At this point, however, to fully understand the role of TAD soils in Pythium suppression, longer term studies will be necessary.

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Table 1. Bacteria, plant pathogens and plants used in this study.

Organism	Characteristics	Source
P. fluorescens	DAPG genotype[a]	
Pf-5	A	(21)
Q2-87	B	(58)
Q8r1-96	D	(48)
5MR2	E	(28)
F113	K	(52)
1M1-96	L	(28)
phL1C2	M	(15)
MVW4-2R	Q	(28)
Plant pathogens	Isolate[b]	
P. abappressorium	20162	(45)
P. attrantheridium	20169	(2)
Pythium spp. *(*aff *echinulatum)*	20131	(44)
P. heterothallicum	90014	(44)
P. irregulare group I	900101	(32)
P. irregulare group IV	20155	(32)
P. paroecandrum	90031	(44)
P. rostratifingens	20172	(6)
P. ultimum	30141	(44)
Plants		
Triticum aestivum cv Penawawa	Spring wheat	

[a] Genotypes were determined by whole-cell repetitive sequence-based (rep)-PCR (38) and restriction fragment length polymorphism analysis (RFLP) of *phl*D^+ (35).

[b] Isolates from the collection of T. C. Paulitz. USDA ARS, Washington State University. Pullman, WA. 99164.

Table 2. Physical, chemical and biological properties of the soils used in this study

Soils	pH	Content (µg/g soil)				Content (%)				Texture	Suppressive to take-all
		NO_3N	NH_4N	P	K	OM	Sand	Clay	Silt		
Quincy	5.3	29.8	0.9	10.3	330	1.01	42.8	10.8	46.4	Loam	+
Quincy virgin	6.8	20.1	1.3	6.8	326	0.98	46.8	4.8	48.4	Sandy loam	-
Lind	5.7	92.8	3.0	4.1	520	1.62	31.2	4.8	64	Silt loam	+
Lind virgin	8	12.5	0.6	9.1	184	0.98	29.2	4.8	66	Silt loam	-

Table 3. Total culturable aerobic heterotrophic bacteria and *Pseudomonas* spp. in the rhizosphere of wheat grown in take-all suppressive and conducive soils artificially infested with *Pythium* spp.

	P. abappressorium[w]		*P. irregulare* group I		*P. irregulare* group IV		*P. ultimum*	
Soil	TCB[x]	TCP[y]	TCB	TCP	TCB	TCP	TCB	TCP
LTAD	9.11a[z]	9.03a	9.50a	9.06a	9.23a	8.88a	9.29a	8.90b
LV	9.15a	8.91a	9.22a	8.74a	8.80a	8.50a	9.38a	8.65b
LTADT	9.17a	8.96a	9.33a	8.54a	9.03ab	8.93a	9.32a	9.24a
LVT	9.60a	9.04a	9.11a	8.68a	8.85a	8.53a	8.76a	8.48b
P value	0.9806	0.7795	0.6857	0.1370	0.0361	0.5926	0.1359	0.0014
QTAD	9.43a	8.96a	9.31a	9.13a	9.22a	8.80a	9.39a	8.62a
QV	9.27a	8.50a	9.01a	8.60b	9.00a	8.64ab	9.41a	8.66a
QTADT	9.47a	9.16a	9.22a	8.83ab	9.32a	8.90a	9.25a	8.65a
QVT	9.26a	8.86a	9.08a	8.65b	9.22a	8.23b	9.14a	8.55a
P value	0.3006	0.1283	0.3641	0.0358	0.4679	0.0186	0.6643	0.9128

[w] Each pathogen was introduced at a rate of 10^3 CFU/g of soil. Infested soils were maintained wet at 15°C for seven days before planting 2-day old pregerminated wheat seeds cv. Penawawa.

[x] Total culturable aerobic heterotrophic bacteria (log CFU/g root) determined by the dilution end-point method with 1/10x TSB^+

[y] Total culturable *Pseudomonas* spp. (log CFU/g root) determined by the dilution end-point method with 1/3x KMB^{+++}

[z] Values are the mean of six repetitions. Means with the same letter or no letter within each set of soils are statistically similar according to Fisher's protected least significant difference test (P=0.05).

Table 4. Inhibition of mycelial growth of *Pythium* spp. by DAPG-producing *P. fluorescens*

		P. abappressorium		*P. irregulare* group I		*P. irregulare* group IV		*P. ultimum*	
		Inhibition index[a]		Inhibition index		Inhibition index		Inhibition index	
Strain	Genotype	1/5 PDA[b]	KMB	1/5 PDA	KMB	1/5 PDA	KMB	1/5 PDA	KMB
Pf-5	A	0.13bc	0.22a	0.05c	0.62a	0.11cd	0.41a	0.04d	0.45 ^{A}
Q2-87	B	0.35a	0.13bc	0.33a	0.41b	0.30a	0.39ab	0.28a	0.27 ^{C}
Q8r1-96	D	0.19b	0.19ab	0.12b	0.38b	0.15bc	0.39ab	0.07c	0.34 ^{B}
5MR2R	E	0.19b	0.14bcd	0.12b	0.29cd	0.17b	0.26c	0.03de	0.14 ^{E}
F113	K	0.34a	0.16abc	0.30a	0.37bc	0.27a	0.39ab	0.23b	0.29 ^{BC}
1M1-R	L	0.12cd	0.11bcd	0.07c	0.28d	0.17bc	0.31bc	0.02e	0.24 ^{CD}
C2phL1	M	0.38a	0.15bcd	0.29a	0.37bc	0.32a	0.34abc	0.28a	0.18 ^{DE}
MVW4-2R	Q	0.08d	0.08d	0.07c	0.27d	0.09d	0.25c	-------[d]	0.17 ^{E}

[a] Inhibition index. Values are means of six repetitions and the experiment was repeated twice. Common letters within each column do not differ significantly (P=0.05) according to Fisher's least significant difference test. In some cases, data were rank transformed before statistical analysis was conducted.

[b] 1/5x potato dextrose agar.

[c] King's B Medium

[d] No inhibition was observed

Table 5. Effect of synthetic DAPG on mycelial growth of *Pythium* spp.

Pythium spp.	CE_{50}[a] µg ml^{-1}	MIC[b] µg ml^{-1}
P. abappressorium	6.0 bc	30
P. attrantheridium	3.8 d	25
P. spp. *(aff. echinulatum)*	6.5 bc	25
P. heterothallicum	4.0 cd	15
P. irregulare group I	9.9 a	30
P. irregulare group IV	7.4 b	25
P. paroecandrum	14.6 a	30
P. rostratifingens	5.0b cd	25
P. ultimum	12.9 a	25

[a] Concentration of 2,4-DAPG required to inhibit by 50 % the radial mycelial growth of *Pythium* spp. CE_{50} were determined using the probit analysis. DAPG doses were log transformed and percentages of inhibition of *Pythium* spp. in comparison with the control were transformed to probits (Hubert, 1980). Means with different letter are significantly different according to Fishers's protected last significant difference (P=0.05). Values are the mean of 6 repetitions and are the result of a representative experiment.

[b] Minimal concentration needed to completely inhibit mycelial growth. The assay was conducted on potato broth amended with 0.8 % agar at pH 6.5. Concentrations of DAPG ranged between 0 and 40 µg ml^{-1} with intervals of 5 µg ml^{-1}. Values are the result of 2 experiments with 6 repetitions per treatment.

Figure 1. Effect of *Pythium* spp. on shoot height of wheat grown in soils suppressive and conducive to take-all from Quincy, WA. Soils, with the exception of the control, were artificially infested with *Pythium* spp. at a rate of 10^3 propagules/g of soil. The experiment was repeated twice, and a representative experiment is shown. Control treatment was not included in the statistical analysis. Each treatment was replicated six times with each pot serving as a replicate. Bars with the same letter within a *Pythium* spp. are not significantly different (P=0.05) according to Fisher's protected least significant difference test. QTAD= Quincy TAD soil, QV= Quincy virgin soil, QTADT= transferred Quincy TAD soil, QVT= transferred Quincy virgin soil.

Figure 2. Effect of *Pythium* spp. on length of first true leaf of wheat grown in soils suppressive and conducive to take-all from Quincy, WA. Soils, with the exception of the control, were artificially infested with *Pythium* spp. at a rate of 10^3 propagules/g of soil. The experiment was repeated twice, and a representative experiment is shown. Control treatment was not included in the statistical analysis. Each treatment was replicated six times with each pot serving as a replicate. Bars with the same letter within a *Pythium* spp. are not significantly different (P=0.05) according to Fisher's protected least significant difference test. QTAD= Quincy TAD soil, QV= Quincy virgin soil, QTADT= transferred Quincy TAD soil, QVT= transferred Quincy virgin soil.

Figure 3. Effect of *Pythium* spp. on shoot height of wheat grown in soils suppressive and conducive to take-all from Lind, WA. Soils, with the exception of the control, were artificially infested with *Pythium* spp. at a rate of 10^3 propagules/g of soil. The

experiment was repeated twice and a representative experiment is shown. Control treatment was not included in the statistical analysis. Each treatment was replicated 6 times with each pot serving as a replicate. Bars with the same letter within a *Pythium* spp. are not significantly different (P=0.05) according to Fisher's protected least significant difference test. QTAD= Quincy TAD soil, QV= Quincy virgin soil, QTADT= transferred Quincy TAD soil, QVT= transferred Quincy virgin soil.

Figure 4. Effect of *Pythium* spp. on length of first true leaf of wheat grown in soils suppressive and conducive to take-all from Lind, WA. Soils, with the exception of the control, were artificially infested with *Pythium* spp. at a rate of 10^3 propagules/g of soil. The experiment was repeated twice, and a representative experiment is shown. Control treatment was not included in the statistical analysis. Each treatment was replicated six times with each pot serving as a replicate. Bars with the same letter within a *Pythium* spp. are not significantly different (P=0.05) according to Fisher's protected least significant difference test. QTAD= Quincy TAD soil, QV= Quincy virgin soil, QTADT= transferred Quincy TAD soil, QVT= transferred Quincy virgin soil.

Figure 5. Effect of *Pythium* spp. on total root length of wheat grown in suppressive and conducive soils from Quincy, WA. Soils, with the exception of the control, were artificially infested with *Pythium* spp. at a rate of 10^3 propagules/g of soil. The experiment was repeated twice, and a representative experiment is shown. Control treatment was not included in the statistical analysis. Each treatment was replicated six times with each pot serving as a replicate. Bars with the same letter within a *Pythium* spp.

are not significantly different (P=0.05) according to Fisher's protected least significant difference test. QTAD= Quincy TAD soil, QV= Quincy virgin soil, QTADT= transferred Quincy TAD soil, QVT= transferred Quincy virgin soil.

Figure 6. Effect of *Pythium* spp. on number of root tips of wheat grown in suppressive and conducive soils from Quincy, WA. Soils, with the exception of the control, were artificially infested with *Pythium* spp. at a rate of 10^3 propagules/g of soil. Experiment was repeated twice, and a representative experiment is shown. Control treatment was not included in the statistical analysis. Each treatment was replicated six times with each pot serving as a replicate. Bars with the same letter within a *Pythium* spp. are not significantly different (P=0.05) according to Fisher's protected least significant difference test. QTAD= Quincy TAD soil, QV= Quincy virgin soil, QTADT= transferred Quincy TAD soil, QVT= transferred Quincy virgin soil.

Figure 7. Effect of *Pythium* spp. on average of root diameter of wheat grown in take-all suppressive and conducive soils from Quincy, WA. Soils, with the exception of the control, were artificially infested with *Pythium* spp. at a rate of 10^3 propagules/g of soil. Experiment was repeated twice, and a representative experiment is shown. Control treatment was not included in the statistical analysis. Each treatment was replicated six times with each pot serving as a replicate. Bars with the same letter within a *Pythium* spp. are not significantly different (P=0.05) according to Fisher's protected least significant difference test. QTAD= Quincy TAD soil, QV= Quincy virgin soil, QTADT= transferred Quincy TAD soil, QVT= transferred Quincy virgin soil.

Figure 8. Effect of *Pythium* spp. on total root length of wheat grown in take-all suppressive and conducive soils from Lind, WA. Soils, with the exception of the control, were artificially infested with *Pythium* spp. at a rate of 10^3 propagules/g of soil. Experiment was repeated twice, and a representative experiment is shown. Control treatment was not included in the statistical analysis. Each treatment was replicated six times with each pot serving as a replicate. Bars with the same letter within a *Pythium* spp. are not significantly different (P=0.05) according to Fisher's protected least significant difference test. QTAD= Quincy TAD soil, QV= Quincy virgin soil, QTADT= transferred Quincy TAD soil, QVT= transferred Quincy virgin soil.

Figure 9. Effect of *Pythium* spp. on number of root tips of wheat grown in in take-all suppressive and conducive soils from Lind, WA. Soils, with the exception of the control, were artificially infested with *Pythium* spp. at a rate of 10^3 propagules/g of soil. Experiment was repeated twice, and a representative experiment is shown. Control treatment was not included in the statistical analysis. Each treatment was replicated six times with each pot serving as a replicate. Bars with the same letter within a *Pythium* spp. are not significantly different (P=0.05) according to Fisher's protected least significant difference test. QTAD= Quincy TAD soil, QV= Quincy virgin soil, QTADT= transferred Quincy TAD soil, QVT= transferred Quincy virgin soil.

Figure 10. Effect of *Pythium* spp. on average of root diameter of wheat grown in take-all suppressive and conducive soils from Lind, WA. Soils, with the exception of the control, were artificially infested with *Pythium* spp. at a rate of 10^3 propagules/g of soil.

Experiment was repeated twice, and a representative experiment is shown. Control treatment was not included in the statistical analysis. Each treatment was replicated six times with each pot serving as a replicate. Bars with the same letter within a *Pythium* spp. are not significantly different (P=0.05) according to Fisher's protected least significant difference test. QTAD= Quincy TAD soil, QV= Quincy virgin soil, QTADT= transferred Quincy TAD soil, QVT= transferred Quincy virgin soil.

Figure 11. Population densities of indigenous DAPG-producing *P. fluorescens* in the rhizosphere of wheat grown in take-all suppressive soils infested and non-infested with different *Pythium* spp. *Pythium* spp. were artificially introduced at a rate of 10^3 propagules/g of soil. Bacterial populations in the rhizosphere of wheat were determined two weeks after sowing and log transformed before data analysis. Values are the mean of six replicates and are expressed as log of the mean (CFU/g of fresh weight of root). Bars with the same letter within a *Pythium* spp. are not significantly different (P=0.05) according to Fisher's protected least significant difference test. QTAD= Quincy TAD soil, LTAD= Lind TAD soil, QTADT= Quincy TAD soil diluted (1:9) with pasteurized Quincy virgin soil. LTADT= Lind TAD soil diluted (1:9) with pasteurized Quincy virgin soil.

Figure 1.

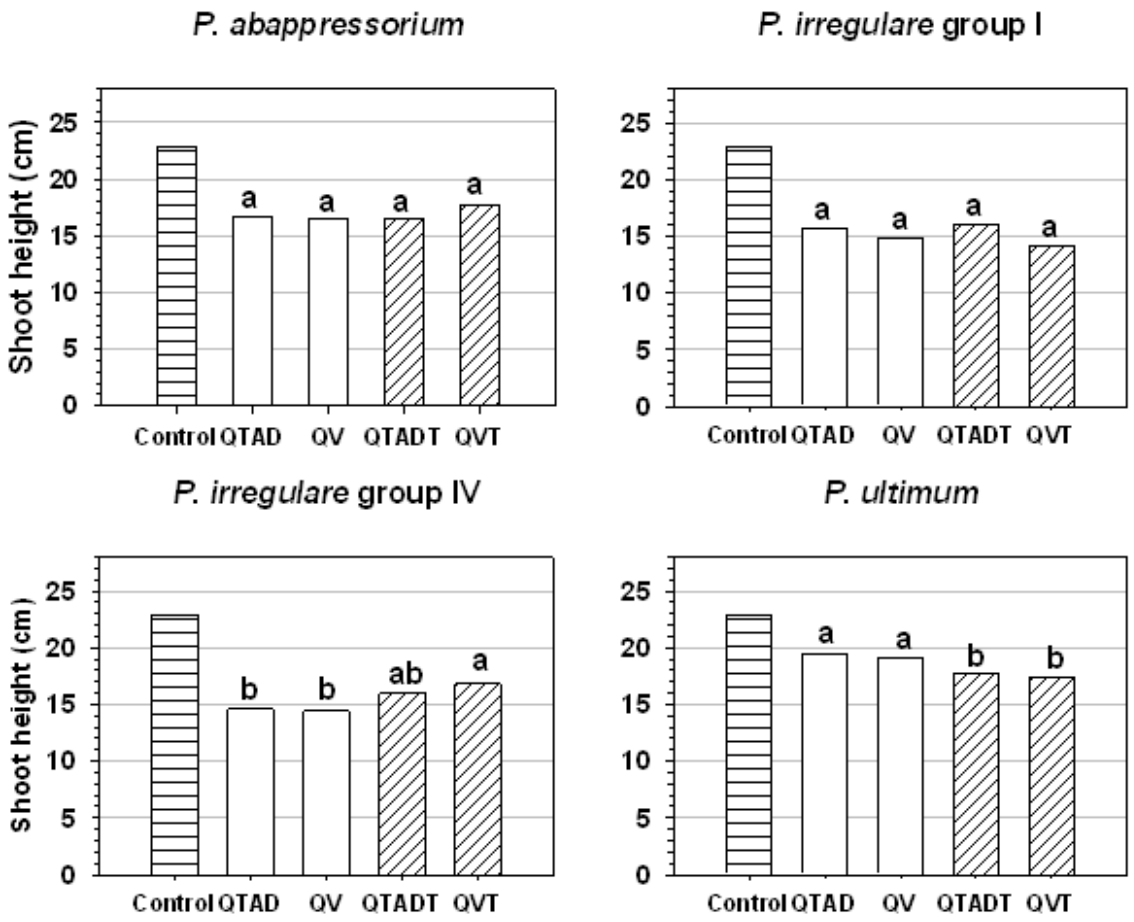

P. abappressorium
P. irregulare group I
P. irregulare group IV
P. ultimum
Shoot height (cm)
0
5
10
15
20
25
a
b
ab
Control
QTAD
QV
QTADT
QVT

Figure 2.

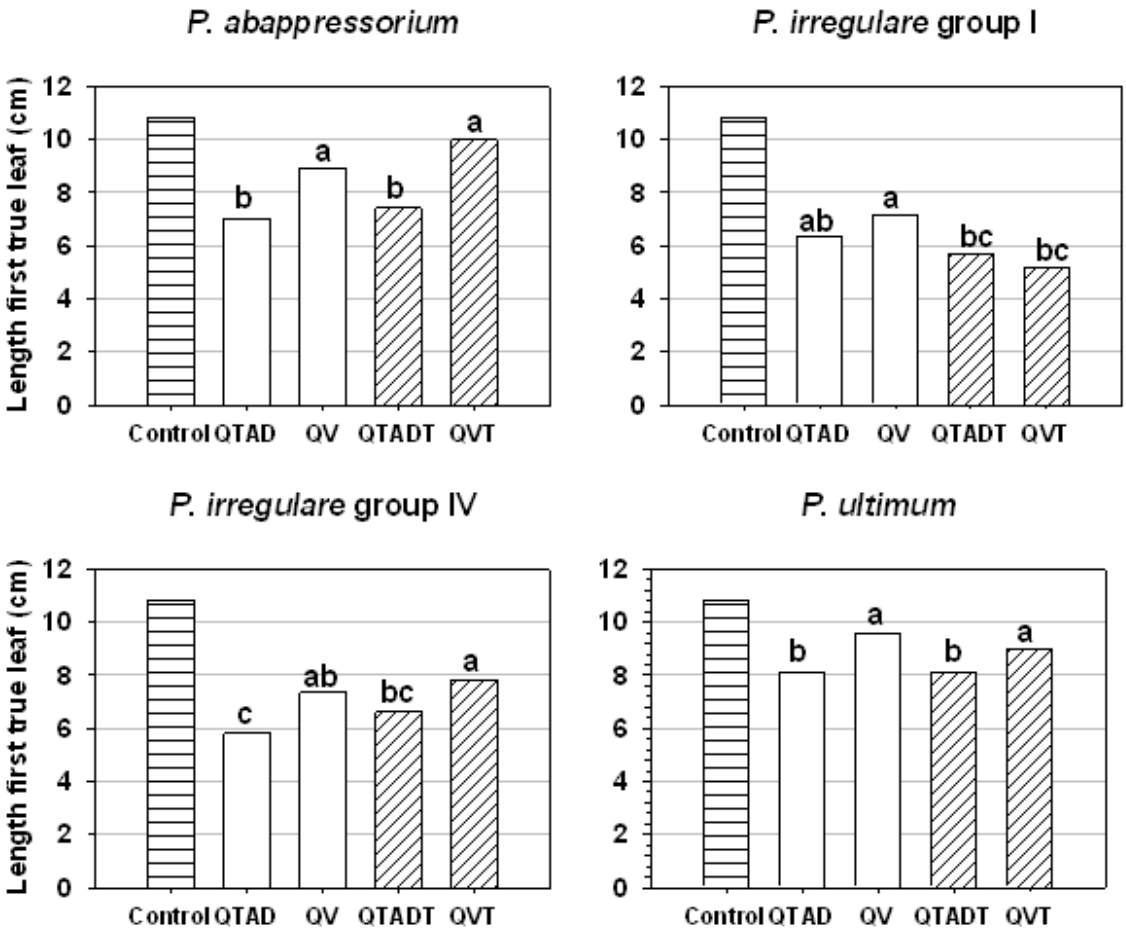

P. abappressorium
P. irregulare group I
P. irregulare group IV
P. ultimum
Length first true leaf (cm)
0
2
4
6
8
10
12
Control
QTAD
QV
QTADT
QVT
b
a
b
a
ab
a
bc
bc
c
ab
bc
a
b
a
b
a

Figure 3.

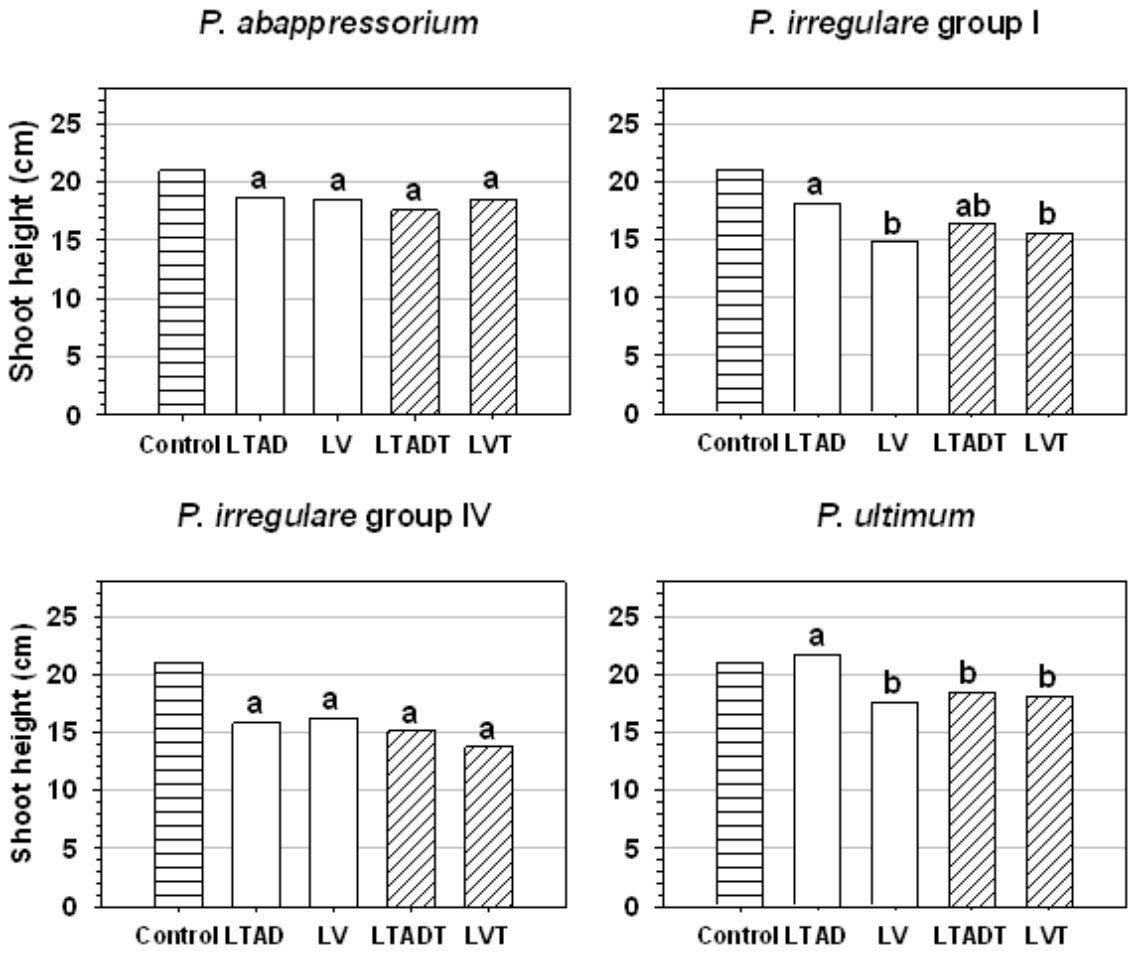

P. abappressorium
P. irregulare group I
P. irregulare group IV
P. ultimum
Shoot height (cm)
0
5
10
15
20
25
a
a
a
a
a
b
ab
b
a
a
a
a
a
b
b
b
Control
LTAD
LV
LTADT
LVT

Figure 4.

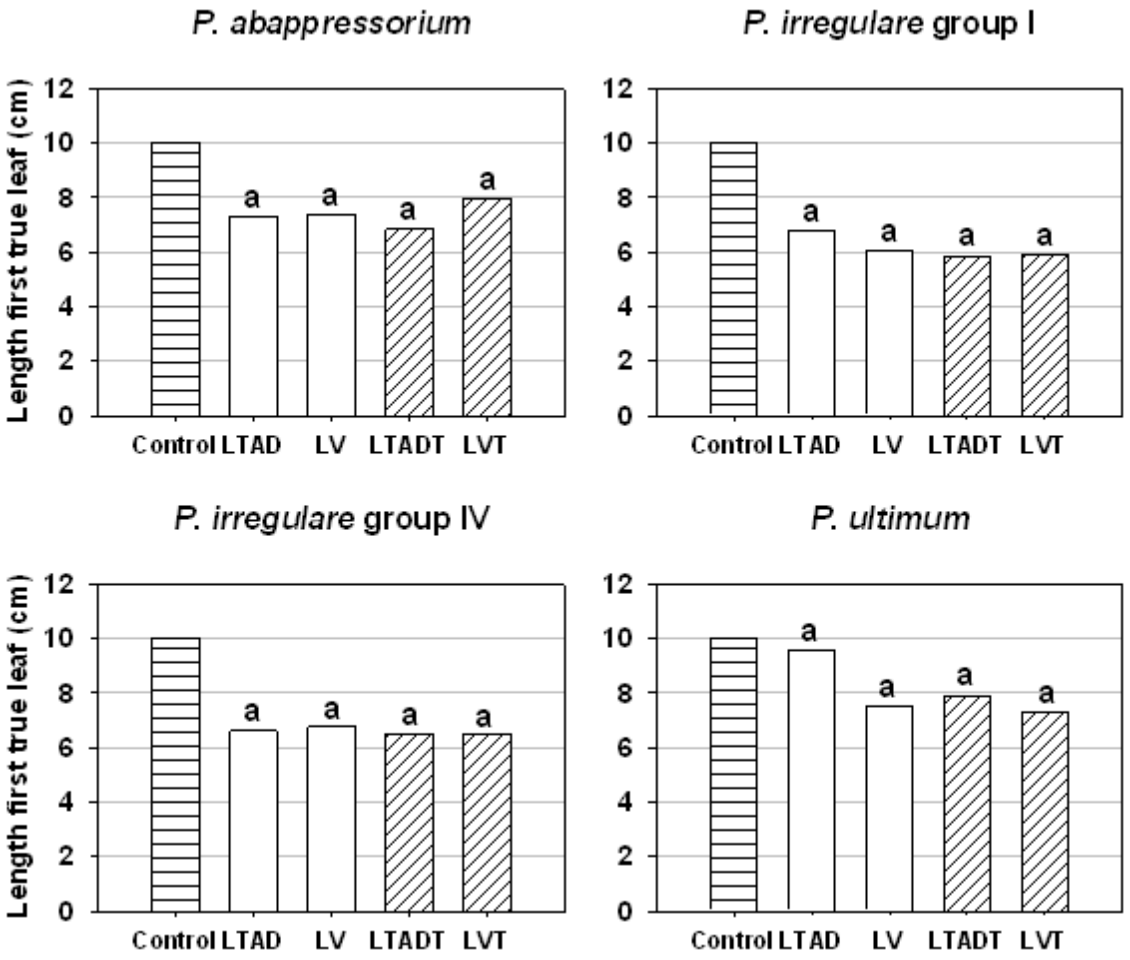

P. abappressorium
P. irregulare group I
P. irregulare group IV
P. ultimum
Length first true leaf (cm)
0
2
4
6
8
10
12
a
Control
LTAD
LV
LTADT
LVT

Figure 5.

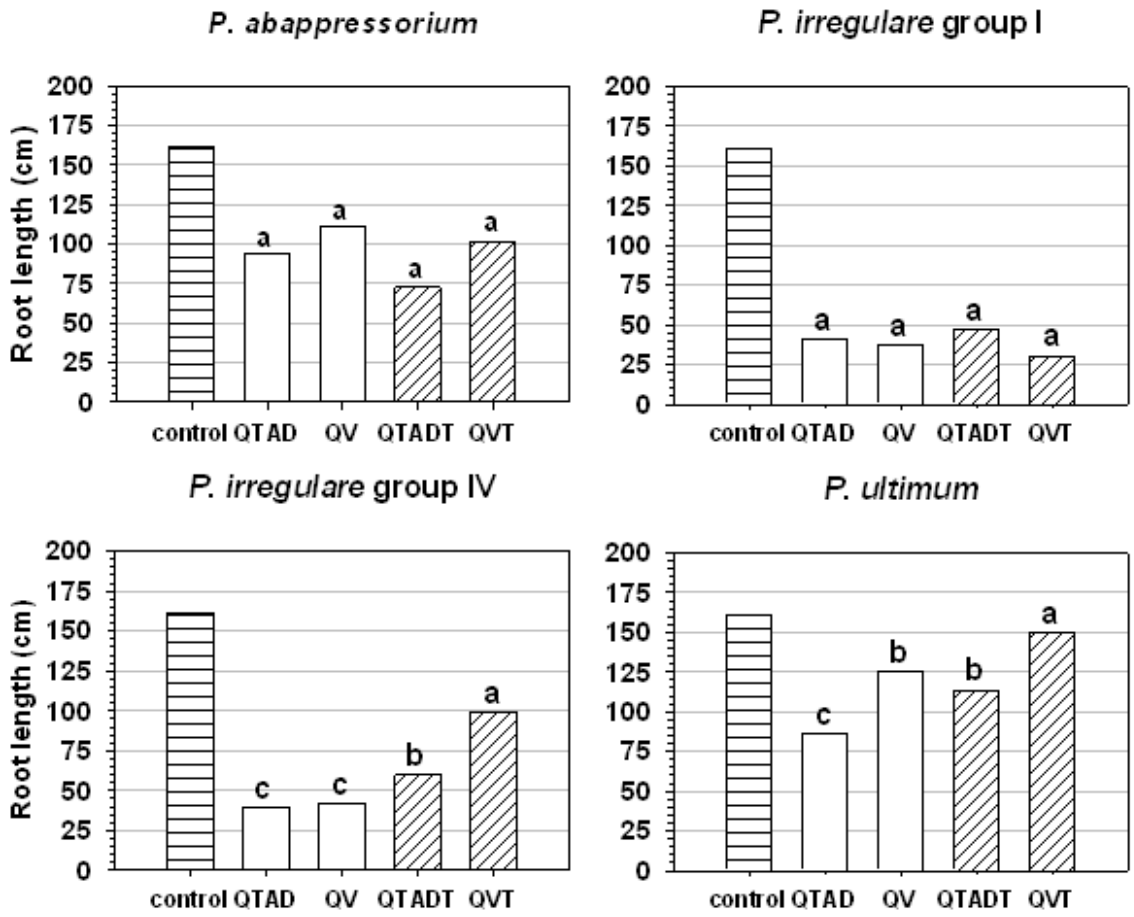

P. abappressorium
P. irregulare group I
P. irregulare group IV
P. ultimum
Root length (cm)
0
25
50
75
100
125
150
175
200
control QTAD QV QTADT QVT
a
a
a
a
a
a
a
a
c
c
b
a
c
b
b
a

Figure 6.

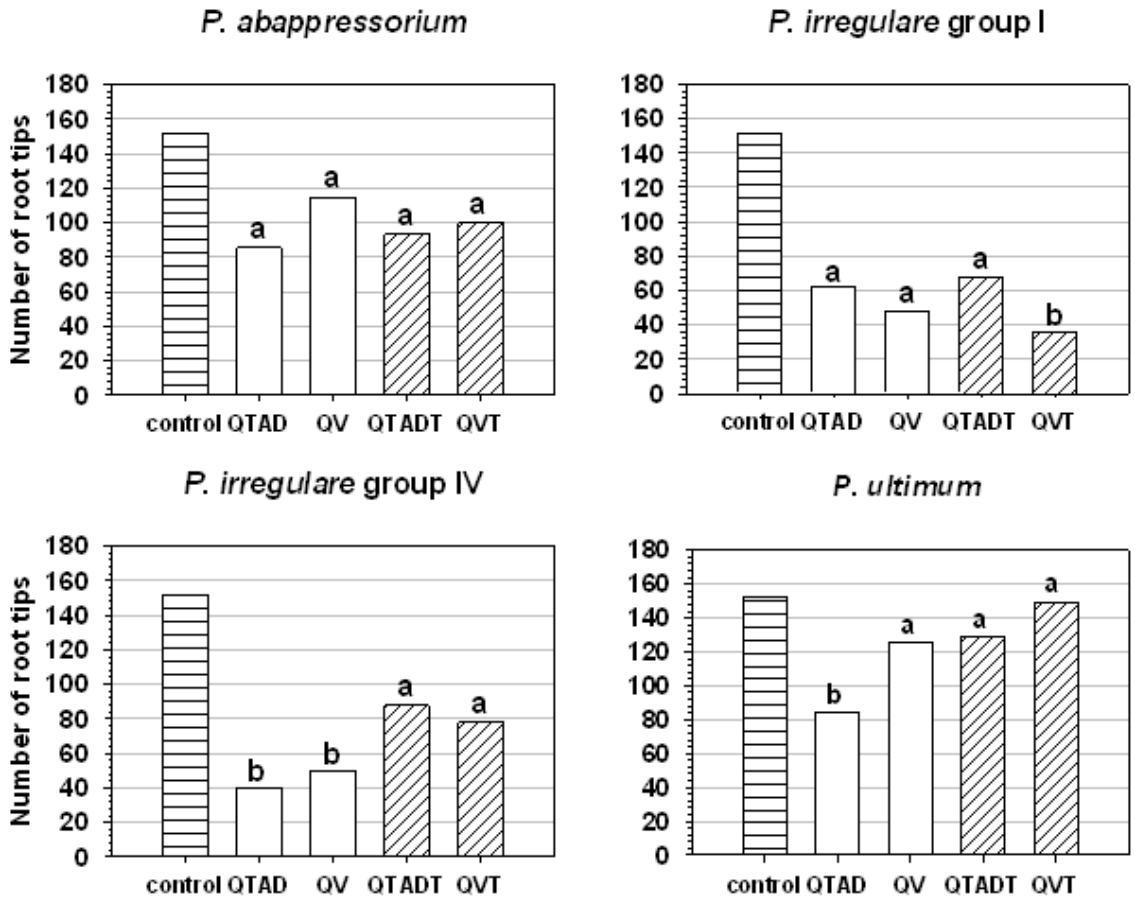

P. abappressorium
P. irregulare group I
P. irregulare group IV
P. ultimum
Number of root tips
0
20
40
60
80
100
120
140
160
180
control
QTAD
QV
QTADT
QVT
a
b

Figure 7.

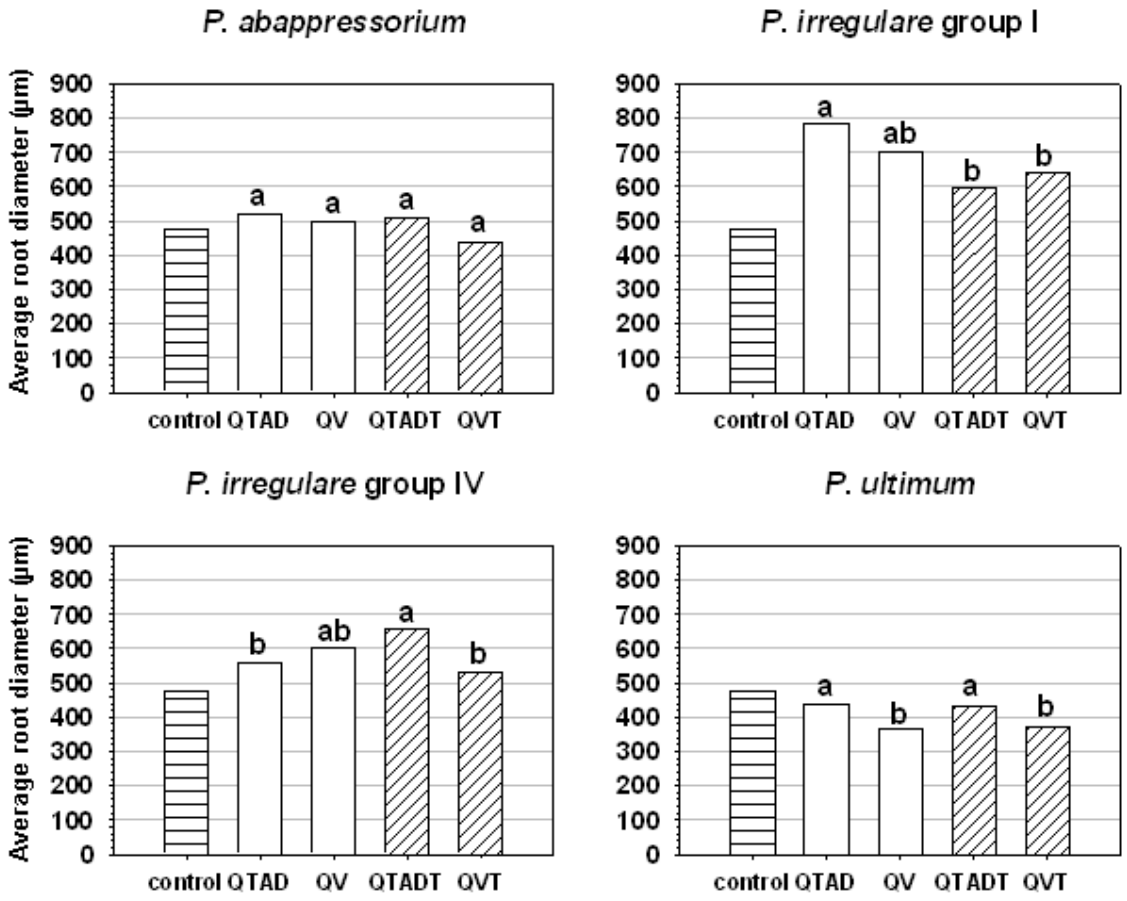

P. abappressorium
P. irregulare group I
P. irregulare group IV
P. ultimum
Average root diameter (µm)
0
100
200
300
400
500
600
700
800
900
control
QTAD
QV
QTADT
QVT
a
ab
b

Figure 8.

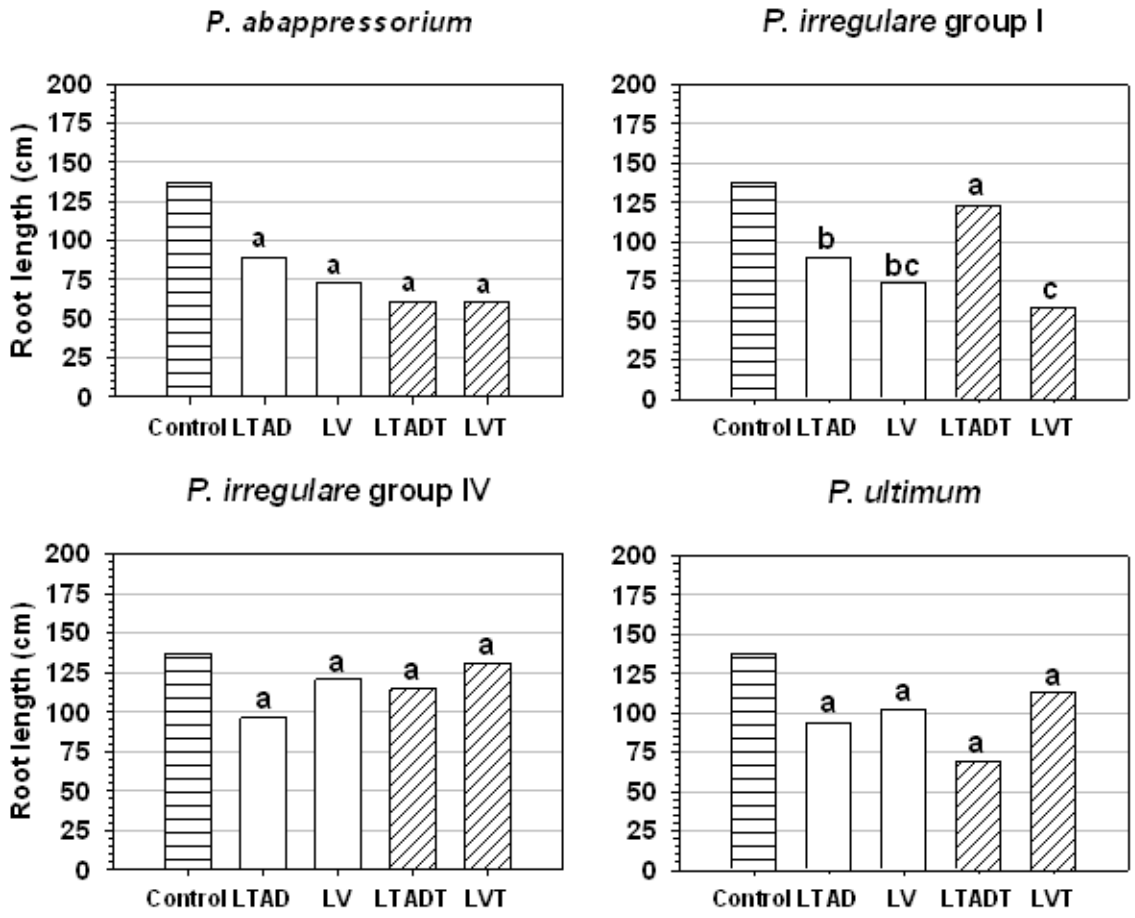

P. abappressorium
P. irregulare group I
P. irregulare group IV
P. ultimum
Root length (cm)
0
25
50
75
100
125
150
175
200
Control
LTAD
LV
LTADT
LVT
a
a
a
a
b
bc
a
c
a
a
a
a
a
a
a
a

Figure 9.

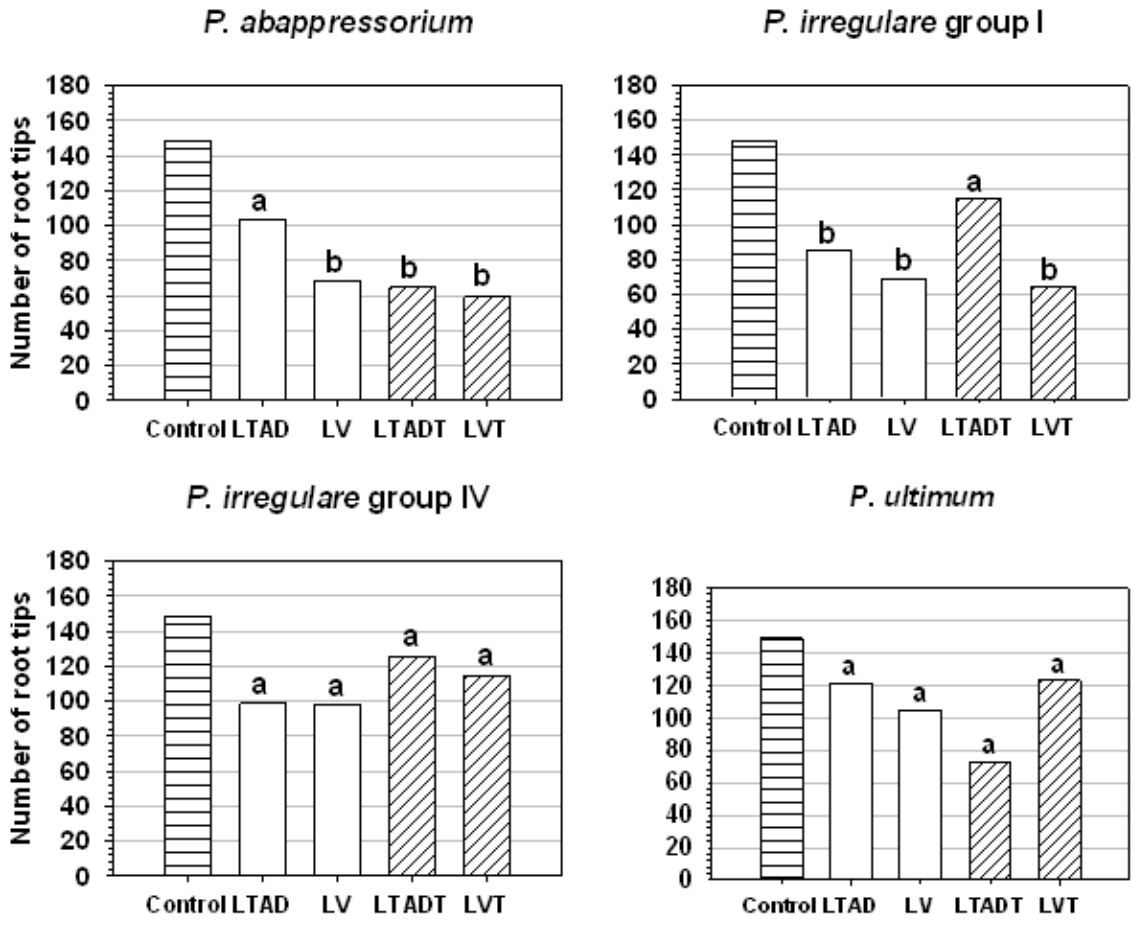

P. abappressorium
P. irregulare group I
P. irregulare group IV
P. ultimum
Number of root tips
0
20
40
60
80
100
120
140
160
180
Control
LTAD
LV
LTADT
LVT
a
b

Figure 10.

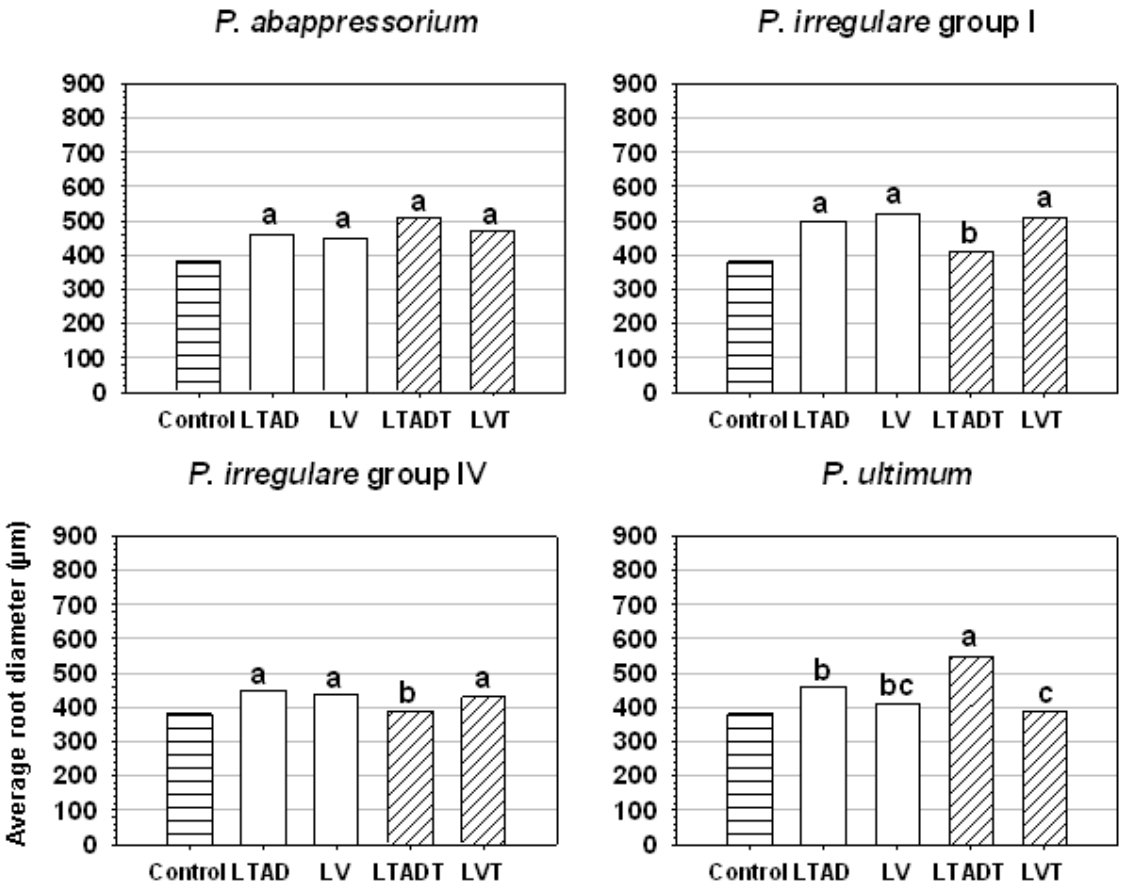
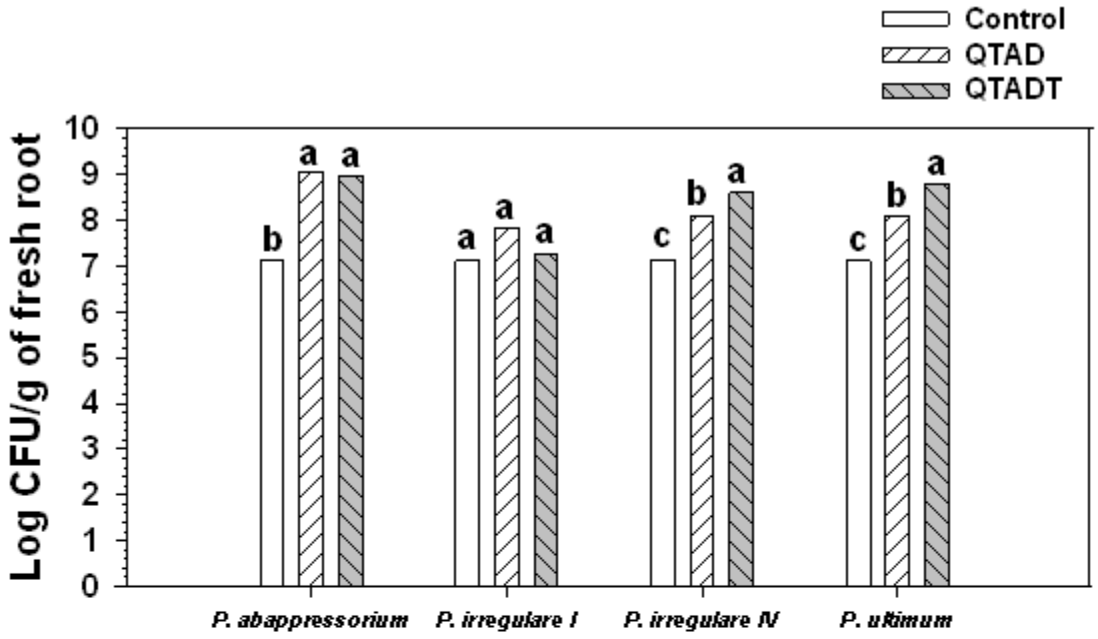
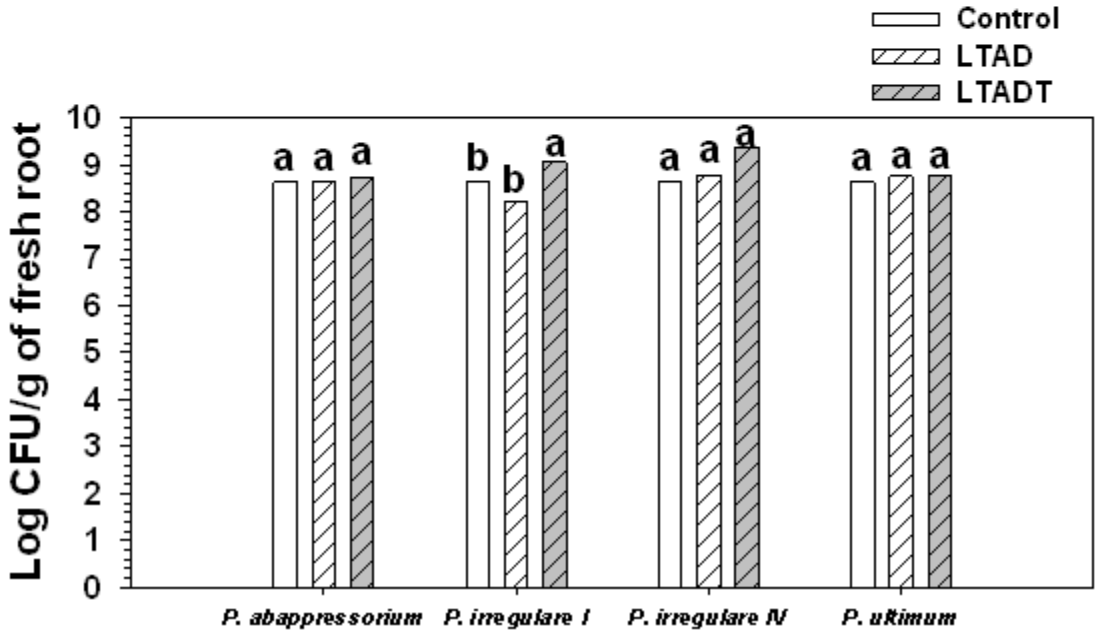

P. abappressorium
P. irregulare group I
P. irregulare group IV
P. ultimum
Average root diameter (µm)
900
800
700
600
500
400
300
200
100
0
Control LTAD LV LTADT LVT
a a a a
a a b a
a a b a
b bc a c

Figure 11.


Control
QTAD
QTADT
Log CFU/g of fresh root
0
1
2
3
4
5
6
7
8
9
10
b
a
a
a
a
a
c
b
a
c
b
a
P. abappressorium
P. irregulare I
P. irregulare IV
P. ultimum


Control
LTAD
LTADT
Log CFU/g of fresh root
0
1
2
3
4
5
6
7
8
9
10
a
a
a
b
b
a
a
a
a
a
a
a
P. abappressorium
P. irregulare I
P. irregulare IV
P. ultimum

CHAPTER 3

Effectiveness of DAPG-Producing *P. fluorescens* Genotypes and Genetically Engineered *P. fluorescens* Strains to Control Pythium Root Rot of Wheat

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Keywords: DAPG genotypes, transgenic pseudomonads, phenazine-1-carboxylic acid, biological control.

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ABSTRACT

Fluorescent pseudomonads are common inhabitants in the rhizosphere of many plants and suppress several soilborne diseases. In this study, a collection of *P. fluorescens* strains belonging to different DAPG genotypes and two genetically engineered *P. fluorescens* strains were tested for their ability to control Pythium root rot of wheat. Inhibition of mycelial growth of *Pythium* spp. by the bacterial strains, plant and root measurements, as well as population sizes of bacterial strains in the rhizosphre of wheat grown in soil infested by *Pythium* spp. were determined. Population sizes of *P. fluorescens* strains from different genotypes, and the transgenic strains, introduced in the experiments as seed coating, resulted in population sizes above log 7 of CFU/g of fresh root in the rhizosphere of wheat grown in soil infested by *P. ultimum* or *P. irregulare*, respectively. The transgenic strains Z30-97 and Z34-97 inhibited the mycelial growth of *Pythium* spp. significantly greater than the strains *P. fluorescens* strains 2-79 and Q8r1-96 in the assays conducted on PDA. A consistent reduction of plant and root measurements was observed due to the presence of *Pythium* spp. infecting the wheat seedlings. In greenhouse experiments none of the bacteria applied as seed coatings resulted in suppression of Pythium root rot of wheat. Plant height, length of the first true leaf, root length, average root diameter, and number of root tips did not differ on wheat seedlings grown from bacterial treated and non-treated seeds.

INTRODUCTION

Pythium spp. are responsible for significant losses in agricultural crops due to causing damping-off and root rot diseases. The main characteristic of infection by *Pythium* spp. is their ability to rapidly infect the germinating seeds within 24 to 48 h after sowing (9). In eastern Washington, root rot of wheat can be caused by more than ten different species of *Pythium* (10). In the seedling stage, Pythium root rot of wheat is characterized by a reduction in emergence, smaller first true leaves, plant stunting and reduction of root length due to destruction of root tips and root hairs (2, 34).

Pseudomonas fluorescens are common inhabitants in soil and in the rhizosphere, and produce a wide array of antibiotics and secondary metabolites active against some plant pathogens. *P. fluorescens* strains have been documented as good biocontrol agents of Pythium diseases in several crops. Howell and Stipanovic (12), showed that *P. fluorescens* strain Pf-5 was able to control Pythium root rot in cotton due to the production of the antibiotic pyoluterin. *P. fluorescens* strain F113 was originally isolated from the rhizosphere of sugarbeet and one of the first strains producing 2,4-diacetylphloroglucinol (DAPG) shown to be effective against Pythium damping-off in sugarbeet (6).

DAPG-producing *P. fluorescens* have been differentiated at the molecular level into 22 DAPG genotypes based on whole cell repetitive sequence based polymerase chain reaction using the BOXA1R primer and restriction fragment length polymorphism (RFLP) and phylogenetic analyses of *phlD*, a key gene involved in the synthesis of DAPG (8, 19, 24, 26, 27, 29). DAPG genotypes differ in antibiotic production,

geographic distribution, host preference and rhizosphere colonization. For instance, strains in genotype A, such as strains Pf-5 and CHA0, produce the antibiotic pyoluterin and hydrogen cyanide besides DAPG (12, 16). With respect to geographic distribution, it is important to note that although DAPG producers play a role in the take-all decline in both Washington, USA, and The Netherlands, the genotypes isolated from these fields differ. Isolates of genotypes B, D, E and L occur in TAD fields in the Washington State and isolates of genotype M and F predominate in Dutch TAD soils (38, 43). Differences among genotypes of DAPG producers also are influenced by the host plant. Landa et al. (17) found that strains of *P. fluorescens* belonging to genotype P and genotype D were predictive of aggressive colonization of the rhizosphere of pea and wheat, respectively. In experiments involving mixtures of strains belonging to different genotypes in a soil, de la Fuente et al. (7) demonstrated that the plant species exerts a significant effect on the outcome of the competition among these genotypes in the rhizosphere.

The antibiotic PCA is the primary determinant of the ability of *P. fluorescens* 2-79 (44) and *P. aureofaciens* 30-84 (37) to suppress take-all of wheat. The expression of the phenazine genes also plays an important role in the ability of these strains to survive in soil and to colonize the wheat rhizosphere in the presence of indigenous microorganisms (23).

Genetically engineered strains, in which foreign genes have been inserted, are being studied as a new resource for controlling soilborne diseases. The main goal in experiments designed to develop bacterial strains with the ability to produce two or more antagonistic compounds is to enhance the efficacy of the biocontrol agent against a pathogen or to broaden its activity against other pathogens. Strains Z30-97 and Z34-97

are recombinant derivatives of *P. fluorescens* Q8r1-96, in which the PCA biosynthetic locus from strain 2-79 was stably inserted into the chromosome. By this insertion, strains Z30-97 and Z34-97 are able to produce DAPG and PCA simultaneously (14), an ability not known in other bacteria in nature.

In a previous experiment, indigenous populations of DAPG producers from soils suppressive to take-all in Washington were unable to reduce Pythium root rot when compared with non-cropped soil lacking DAPG producer populations (R. Allende-Molar, T. C. Paulitz, and D. M. Weller, unpublished data). In this study, we selected DAPG-producing *P. fluorescens* strains belonging to different genotypes to determine their effect when introduced as wheat seed coatings on Pythium root rot of wheat. Additionally, we tested transgenic *P. fluorescens* strains Z30-97 and Z34-97 (1, 14) for their ability to suppress Pythium root rot of wheat. The specific objectives of this study were to: i) evaluate different *P. fluorescens* strains belonging to different genotypes in the suppression of Pythium root rot caused by *P. ultimum* and, ii) determine whether the simultaneous production of the antibiotics DAPG and PCA by the transgenic strains Z30-97 and Z34-97 influences their biocontrol activity against Pythium root rot of wheat.

MATERIALS AND METHODS

Soil and microorganisms. Soil was collected from the upper 30-cm layer in an undisturbed non-cropped field near Quincy, WA. The physical, chemical and microbial characteristics of Quincy soil have been described (39). Soil was air-dried, sieved through

a mesh screen (0.5 cm) and then pasteurized (30 min 60°C) before use. Wheat (*Triticum aestivum* L.) cv. Penawawa was used throughout this study.

All bacterial strains used in this study were rifampicin-resistant mutants of the wild type strains and are described in Table 1. Strains Z30-94 and Z34-97 are also kanamycin resistant (14). Bacterial strains were cultured on King's medium B (KMB) agar supplemented with the appropriated antibiotics. Rhizosphere populations of DAPG-producing and transgenic bacteria in the wheat rhizospere were assessed on 1/3x KMB supplemented with cycloheximide (100 µg/ml), ampicillin (40 µg/ml), cloramphenicol (13 µg/ml), and rifampicin (100 µg/ml) (KMB^{+++rif}).

Production of *Pythium* spp. inoculum. Inoculum of *P. ultimum* and *P. irregulare* was produced in mason jars containing a Ritzville silt loam agricultural soil collected from the Washington State University Dryland Experiment Station in Lind, WA. A 0.5 cm hole was drilled in the lids of the mason jars, and a 70-mm diam. filter disk (Fungi Perfecti, Corvallis, OR) was placed inside the lid to maintain air exchange and sterility. Soils were amended with 1% ground rolled oats (Old Fashioned Quaker Oats, Chicago, IL), added to the jars and then autoclaved twice (10). *P. ultimum* or *P. irregulare* was transferred to the jars in the form of PDA plugs (half of a petri plate 80 x 15 mm cut in 1-cm square). Sterile distilled water (50 ml) was added to the jars 48 h before the PDA plugs were added. The jars were shaken and incubated for 3 weeks at room temperature (approximately 25°C), under the light. Prior to using the inoculum, propagule density in the mason jars was assessed by dilution plating on *Pythium* selective medium (30).

Seed coating. Wheat seeds were coated with the bacterial strains as described by McSpadden Gardener et al., (28). Briefly, bacterial strains were grown on KMB agar for 48 h at 25 °C. Bacterial cells were washed twice in water by centrifugation (10,000 x g, 5 min) and suspended in sterile distilled water. Cell densities were determined spectrophotometrically (Dynatech MR5000, Dynatech Laboratories, Burlington, MA) at an absorbance of 600 nm (A_{600}), diluted in water and added into 500 µl of 1 % methylcellulose suspension to give approximately 10^4 colony forming units (CFU) per seed. Then, bacterized seeds were placed on wet germination paper for 48 h in the dark before being sown.

In vitro assay for inhibition of mycelial growth by DAPG-producing *P. fluorescens* strains. Sensitivity of *Pythium* spp. to the antibiotics produced by the fluorescent pseudomonads 2-79 and Q8r1-96, and transgenic strains Z30-97 and Z34-97 was tested by an in vitro assay based on inhibition of mycelial growth. The assays were conducted on homemade PDA. The pH of the media was adjusted to 7.0 before autoclaving. Aliquots (3 µl) of each bacterial strain (OD_{600} nm = 0.1, aprox. 3 x 10^5 CFU) were placed at equidistant spots (3 cm) from the center of the plate. Sterile distilled water (SDW) was spotted as a control. Agar plugs (4 mm in diameter) of each of the four *Pythium* spp. actively growing in 1/5x PDA were individually placed in the center of the plate 48 h later. Plates were incubated at room temperature in the dark. The assay was ended when fungal colonies in the treatment control reached the spot were SDW was spotted.

A modification of the inhibition index (I): I = IN – T described by Ownley et al. (33) was used to determine inhibition of mycelial growth of the *Pythium* spp. by the

bacterial strains. Briefly, the inhibition index is obtained by measuring the inhibition zone (IN) distance from the edge of the bacterial colony to the growing edge of the fungus and divided by the total distance (T) from the edge of the agar plug to the edge of the bacterial colony. Resulting data are between index 0 and 1 meaning percentage of inhibition compared with a control.

***Pythium* suppression assay.** Plastic cones (16 cm high, 2.5 cm wide) (Stuewe & Sons, Corvallis, OR) were plugged with cotton to prevent soil and moisture loss. Each cone was filled with approximately 55 g of dry soil infested previously with inoculum of the pathogen increased in mason jars. *P. ultimum* was introduced into the soil at a rate of 10^3 propagules/g of soil and then 0.5 % of ground rolled oats was amended to serve as food base for *P. ultimum*. Control treatment consisted of soil without inoculum. Twenty ml of water were added to the cones and the cones were placed in racks and maintained in a greenhouse at 15 °C with 12 h photoperiod. Soils in the cones were maintained wet by adding tap water as needed. After 7 days, two-day-old pre-germinated bacterized wheat seeds were sown and covered with a 1-cm layer of Quincy virgin non-infested soil. Treatments were arranged into the racks as a randomized complete block design. Tubes and racks were covered with a plastic film to maintain humidity until seedling emergence. A fertilizer solution 10 ml (0.93 g/L Miracle GRO, Port York) was added twice per week. Seedlings were allowed to grow for 2 weeks and then harvested to analyze bacterial colonization in the rhizosphere and disease suppression of Pythium root rot.

Studies of the biocontrol activity of the transgenic *P. fluorescens* strains were conducted in pots (8 cm wide, 7.5 cm high). *P. irregulare* was introduced into the soil at

a rate of 10^3 propagules/g of soil and then 0.25% ground rolled oats (w/w) were amended to serve as a food base for the introduced inoculum (11), to stimulate the growth and establishment of the *Pythium* inoculum. Tap water (40 ml) was added into each pot, and then pots were covered with a plastic bag to prevent desiccation and maintained for 7 days in a growth chamber at 15°C prior to planting. Eight two-day-old pre-germinated wheat seeds (cv. Penawawa) were then sown in each pot containing 200 g of soil. Water and fertilizing was conducted as described above. Treatments were arranged in a completely randomized design with each treatment replicated 6 times, and a single pot, serving as a replicate.

Plant and root measurements. Two or three plants per treatment replicate were randomly selected for root measurements. Roots of the plants were washed free of soil and debris using a stream of tap water and then were digitally scanned using a Hewitt-Packard Scanjet 5370C scanner and saved as TIF files. Root scans were analyzed using WinRhizo software (Regent Instruments Inc. Quebec, Canada). This software calculates total root length, average root diameter, and number of root tips. Shoot height and length of the first true leaf were determined on at least six plants randomly selected from each treatment replicates.

Determination of population densities of introduced DAPG producers, transgenic bacteria and total bacteria in the wheat rhizosphere. At the end of each experiment, one plant from each treatment replicate was randomly selected to determine the population size of the introduced bacteria in the rhizosphere. Root systems were removed from soil and carefully shaken to remove loosely adhering soil, leaving only tightly adhering rhizosphere soil. Roots were excised from the plants and all roots from a

single plant were placed into a 50-ml screw-cap centrifuge tube containing 10 ml of sterile distilled water. The tube was shaken for 1 min on a Vortex mixer, and sonicated in an ultrasonic cleaner (Bransonic 521, Branson, Shelton, CT) for one minute, essentially as described by Landa et al. (18). Population densities of DAPG producers were estimated using the PCR-based dilution-endpoint assay (18, 28). An aliquot (100 µl) of the root wash was serially diluted (1:3) in a 96-well microtiter plate pre-filled with 200 µl of sterile distilled water per well, and an aliquot (50 µl) of each dilution series was transferred to a 96-well plate containing fresh 1/3x KMB^{+++rif} broth. Microtiter plates with 1/3x KMB^{+++rif} were incubated at room temperature (approximately 25°C) in the dark and bacteria in each well was assessed spectrophotometrically (Dynatech MR5000, Dynatech Laboratories, Burlington, MA) after 96 ± 4 h. An absorbance at 600 nm (OD_{600}) of ≥ 0.07 was scored as positive for growth (28). The terminal dilution culture (TDC) is the greatest dilution (well) showing bacterial growth. Aliquots from wells showing growth were tested for the presence of specific genotypes of DAPG producers by PCR analysis as described below, and population densities were determined on the basis of the last well where the *phl*D signal was detected.

Population densities of the introduced transgenic strains were enumerated by a modification of the PCR based dilution endpoint assay proposed by Validov et al. (45). The modified assay includes an additional step in which bacteria are selected first on 1/3x KMB^{+++rif} and after 72 h of growth, bacteria are transferred into a new plate containing 1/3x KMB^{+++rif} amended with kanamycin (25 µg/ml) to check for cross contamination by the non-transgenic strains.

Bacterial growth was assessed after 72 h with and $OD_{600} \geq 0.07$ considered positive. Aliquots of TDCs were used to verify the identity of the inoculant strains by PCR analysis. A portion of the *phlD* gene present in strains Q8r1-96, Z30-97, and Z34-97 was amplified with the primers B2BF and BPR4 (28), and a portion of the *phz* locus present in strains 2-79, Z30-97, and Z34-97 was amplified by the primers PCA2a and PCA3b (38). The resulting PCR amplification products were separated in a 1.5% agarose gel in 0.5x tris-borate-EDTA (TBE) buffer at 125 V for 2 h. Banding patterns were visualized by ethidium bromide staining and scored by comparison to a 1 Kb bp ladder.

The population density of total heterotrophic culturable bacteria in each sample was determined by transferring aliquots (50 µl) from the serially diluted (1:3) root washings into a 96-well microtiter plate containing 1/10x TSB^{+}. Microtiter plates were incubated at room temperature in the dark, and bacterial growth was assessed spectrophotometrically as described above, after 48 ± 4 h. The terminal dilution in the microplate showing positive growth ($OD_{600} \geq 0.07$) was used to calculate the total population of culturable bacteria in a sample.

Statistical analysis. Data were analyzed by using the Statistix program (version 8, Analytical Software, Tallahassee, FL). Treatments in the disease suppression assays were arranged in the growth chamber as a completely randomized block design or completely randomized design for *P. ultimum* and *P. irregulare* experiments, respectively. One-way analysis of variance was conducted to test for differences between treatments, because no effect of blocks was observed. Bacterial population data were log transformed before statistical analysis. Significant differences between means were determined using Fisher's protected least significant difference. In vitro assays were

arranged in a randomized complete design and data were rank transformed before statistical analysis. When no transformation was available, significant differences between means were determined by the Kruskal-Wallis test.

RESULTS

In vitro assay for inhibition of mycelial growth by genetically engineered DAPG-producing *P. fluorescens* strains. In vitro assays conducted on homemade PDA showed that all strains, with the exception of strain 2-79, inhibited the mycelial growth of *Pythium* spp. (Fig. 1). The transgenic strains Z30-97 and Z-34-97 showed similar mycelial inhibition indexes against all *Pythium* spp. tested. Inhibition indexes of the transgenic strains were significantly greater (P=0.05) than those for strain Q8r1-96. *P. irregulare* group I and *P. ultimum* were less sensitive to effects of the strains tested, as indicated by smaller inhibition indexes as compared to *P. abappressorium* and *P. irregulare* group IV. Isolate of *P. irregulare* group IV was the most sensitive as showed by the highest inhibition indexes.

Determination of population densities of DAPG producers, transgenic and total bacteria in the wheat rhizosphere. Populations of total culturable aerobic heterotrophic bacteria in the rhizosphere of wheat treated with different genotypes ranged between log 9.04 and log 9.45 of CFU/g of fresh root, and were not significantly different (P=0.05). Although the population size of total aerobic heterotrophic bacteria in the rhizosphere of wheat grown in non-infested soil was the lowest, no significant

differences were observed between populations in the rhizosphere of wheat grown in infested and non-infested soil (Table 2). Population densities of DAPG producers in the rhizosphere of wheat grown in soil infested with *P. ultimum* ranged between log 7.79 and log 8.52, and were not significantly different (P=0.244) (Table 2). Population of the non-DAPG producer strain 2-79 was log 8.13 CFU/g of fresh root and was similar to the population sizes of the DAPG producers in the rhizosphere (P=0.244) (Table 2).

In the experiment of Pythium root rot suppression by transgenic *P. fluorescens* strains, the population densities of total culturable aerobic heterotrophic bacteria were significantly different (P=0.002) in the rhizosphere of wheat grown in infested soil than the density of aerobic heterotrophic bacteria in the wheat rhizosphere grown in the non-infested soil. Population densities of total aerobic heterotrophic bacteria in the rhizosphere of wheat treated with bacterial strains were similar to those in the untreated seeds grown in the soil infested by *P. irregulare* (Fig. 2).

The population densities of bacterial strains introduced as seed coating were above log 8 CFU/g of fresh root weight in the wheat rhizosphere, and were not significantly different (P=0.907) across the treatments (Fig. 2). Introduced bacteria were not detected in the rhizosphere of non-treated wheat grown in infested and non-infested soils.

***Pythium* suppression by DAPG-genotypes and transgenic strains of *P. fluorescens*.** Plant and root measurements were affected when plants were grown in infested soil. *Pythium ultimum* caused reductions in the length of both shoot height and first true leaf. Analysis of data obtained by using the software WinRhizo showed that plants grown in infested soil showed consistently shorter lengths, wider root diameters

and smaller numbers of root tips (Table 2) than wheat grown in non-infested soil. Shoot height and length of the first true leaf were not significantly different (P=0.167 and P= 0.685, respectively) on plants grown from seeds treated with DAPG producers as compared with non-treated seeds sown in the infested soil. When plants were treated with either the DAPG-producers or 2-79, means of number of root tips and root length were similar to the means of these parameters on the non-treated plants. There were significant differences (P=0.013) in average root diameter between treatments; root diameters from seedlings of wheat treated with strain Q2-87 were significantly (P=0.013) smaller than the root diameters from the infested control. Similar results were obtained when the experiment was repeated.

A consistent reduction in plant and root measurements was observed in plants grown in soils infested by *P. irregulare* (Fig. 3). Treatment of wheat seeds with the transgenic strains Z30-97 and Z34-94 did not reduced root infection caused by *P. irregulare*. The shoot height of plants treated with the bacterial strains 2-79, Q8r1-96, and Z30-97 was not significantly different as compared with the untreated control. In contrast, seedlings of wheat treated with the Z34-97 strain were significantly (P=0.022) shorter than untreated seedlings. The length of the first true leaf of wheat seedlings grown in *Pythium*-infested soil showed a consistent reduction in size as compared with seedlings in non-infested soil. The length of the first true leaf of seedlings of wheat treated with the bacterial strains was not significantly (P=0.114) different from the length of first leaf of non-treated wheat seeds grown in *Pythium*-infested soil. The shoot fresh weight of seedlings of wheat treated with bacterial strains was not significantly (P=0.085) different

from the weight of seedlings of the non-treated plants grown in *Pythium*-infested soil (Fig. 3).

The root length of seedlings of wheat treated with the bacterial strains 2-79, Q8r1-96, Z30-97, and Z34-97 were not significantly different from seedlings of non-treated seeds grown in *Pythium* infested soil (Fig. 4). Seedlings of wheat treated with strain Z34-97 had significantly (P=0.007) fewer number of root tips as compared with the seedlings of wheat treated with the other bacterial strains. Measurements of root diameter showed that seedlings of wheat treated with strain Z34-97 were significantly (P=0.021) greater from the non-treated control grown in infested soil; other bacterial treatments were not statistically different from the non-treated control.

DISCUSSION

In our study, we assessed the ability of different genotypes of DAPG producers and transgenic *P. fluorescens* strains that produce both DAPG and PCA to suppress Pythium root rot when applied to wheat seeds.

The recombinant strains Z30-97 and Z34-97 were significantly more inhibitory of mycelia growth of *Pythium* spp. than parental strain Q8r1-96 in in vitro assays conducted on homemade PDA (Fig. 1). In contrast, strain 2-79, the source of the PCA genes, did not have any effect on the mycelia growth of the *Pythium* spp. tested. The greater inhibition of *Pythium* spp. by the transgenic strains Z30-97 and Z34-97 were due to the enhanced

DAPG and PCA production in the transgenic strains as compared to what occurs in strains Q8r1-96 and 2-79 (14).

Biocontrol experiments were conducted in pasteurized soil which provided ideal conditions for promoting rhizosphere colonization and antibiotic production by the introduced strains. It also eliminated interference from other soilborne pathogens. Under these ideal conditions, seedlings from non-treated seed and seeds coated with different genotypes of DAPG producing *P. fluorescens* grown in soil infested with *P. ultimum* did not differ significantly in the amount of Pythium root rot on the basis of measurements of shoots and roots (Table 2). These findings support our earlier study that showed that indigenous populations of DAPG producers in TAD soils did not suppress Pythium root rot.

Transgenic strains Z30-97 and Z34-97 were then tested for activity against Pythium root rot. Shoot fresh weight and length of first true leaf from seedlings of wheat treated with the recombinant strains did not show significant differences (P=0.08, and P=0.11, respectively) as compared to seedlings from non-treated seeds grown in soil infested by *P. irregulare* (Fig. 3). Similar results were observed by direct root measurements (Fig. 4). These results are similar to those of Huang et al., (14) that showed a detrimental effect in seedlings of wheat treated with the recombinant strain Z34-97; shoot heights were smaller and the average diameter of roots was wider than in seedlings from non-treated seeds grown in soil infested with *P. irregulare*. Strain Z34-97 was previously shown to have detrimental effect on wheat seedlings in the field possible due to very high production of DAPG and PCA (D. M. Weller, K. L. Schroeder, L. S. Thomashow, and R. J. Cook, unpublished data).

Our results showing that neither wild-type nor transgenic DAPG producers controlled Pythium root rot differed from the findings of other that DAPG-producing *P. fluorescens* strains suppress Pythium diseases in several crops when applied as seed treatments (6, 12, 15, 32, 41).

One reason for the differential effects on *Pythium* may be that the bacteria and antibiotics are phytotoxic on some hosts. For example, Naseby and Lynch (31) coated pea seeds with strain F113 at densities as high as 2.8 x 10^8 CFU/seed and obtained reductions of Pythium damping-off without detrimental effects on the seedlings. In contrast, when wheat seeds were coated with log 10^5 or higher concentrations of the recombinant strains Z30-97 and Z34-97, wheat seed germination was reduced and root and shoot stunting was observed (14). A modified strain CHA0 which overproduced pyoluterin was phytotoxic on cress and sweet corn (25).

A second reason for the inability of DAPG producers to suppress some Pythium diseases is the differential sensitivity of the propagules of *Pythium* inoculum. de Souza et al., (42) found that zoospores were the most sensitive propagule of *P. ultimum* var. *sporangiferum* to DAPG. However, the isolates of *P. irregulare group I* and *P. ultimum* used in this study do not produce zoospores and very rarely produce sporangia (36). The inoculum of *Pythium* spp. used to infest the soils included mostly mycelia, in addition to oospores and hyphal swellings that were colonizing the ground oats (11). Furthermore, the addition of ground rolled oats to the soil to enhance the establishment of the introduced *Pythium* spp., probably increased the inoculum of *Pythium* spp. in the soil.

The ability of a biocontrol agent to colonize the root is an essential requisite for biocontrol of soilborne diseases (20, 45). Soilborne pathogens attacking the roots can

affect both the ability of potential biocontrol agents to colonize the roots of the host and the antibiotic production. Infection of wheat roots by *Pythium* spp. resulted in significantly smaller population densities of *P. fluorescens* strain 2-79 than those on non-infected roots (22). *P. ultimum* repressed the expression of genes in *P. fluorescens* F113 affecting its root colonization ability on sugarbeet (5). The soilborne pathogen *Fusarium oxysporum* f. sp. *radicis-lycopersici* produces fusaric acid, a fungal toxin, which completely abolishes the synthesis of DAPG in *P. fluorescens* CHA0 (4). In our study, infection by *P. ultimum* did not lead to significant differences in total culturable aerobic heterotrophic bacteria in the rhizosphere of seedlings grown from bacterial treated and non-treated seeds (Table 2, Fig. 2). In addition, population densities of the introduced bacteria were similar in the rhizosphere of seedlings grown from treated seeds (Table 2, Fig. 2). In the experiments using the recombinant strains Z30-97 and Z34-97, population densities of total culturable aerobic heterotrophic bacteria were significantly different (P=0.002) in the seedlings grown in the soils infested by *P. irregulare* as compared with seedlings grown in the non-infested soil (Fig. 2); however, population densities of the introduced bacteria in the rhizosphere were not significantly different (P=0.907). Thus, the inability of the DAPG-producing *P. fluorescens* strains to colonize the wheat rhizosphere was not the reason of the failure of the biocontrol, since population densities of introduced bacteria were above log 7 CFU/g of fresh root. However, we think that the failure of the DAPG-producing and transgenic *P. fluorescens* strains to control Pythium root rot of wheat can be attributed to the timing of antibiotic production *in situ* which lags behind infection by the pathogen. *Pythium* spp. begin their parasitic invasion of wheat

seeds within 24-48 h after planting (3, 9). The ability of the strain to produce the antibiotic in the presence of the pathogen is a topic of current study.

In conclusion, evidence is presented in this study that under our experimental conditions *P. fluorescens* strains belonging to different DAPG genotypes and transgenic strains Z30-97 and Z34-97 were not able to reduce Pythium root rot of wheat caused by isolates of either *P. ultimum* or *P. irregulare*. However, it cannot be ruled out that DAPG producers and the transgenic *P. fluorescens* strains could control infections of *Pythium* spp. other than the isolates evaluated in these experiments or suppress Pythium diseases of different plants.

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Table 1. Bacterial strains and pathogens used in this study.

Strain/isolate	Description	Reference
P. fluorescens		
Pf-5	Genotype A	(132)
Q2-87	Genotype B	(45)
Q8r1-96	Genotype D	(37)
F113	Genotype K	(39)
FFL3R49	Genotype T	(19)
2-79[a]		
Transgenic *P. fluorescens*		
Z30-97	Produces DAPG and PCA[b]	(14)
Z34-97	Produces DAPG and PCA	(14)
Pathogens	Isolates[c]	
P. abappressorium	20162	(35)
P. irregulare group I	900101	(21)
P. irregulare group IV	20155	(21)
P. ultimum	30141	(34)

[a] 2-79 produces PCA but not DAPG. It was the source of the PCA genes in Z30-97 and Z34-97

[b] Phenazine-1- carboxylic acid.

[c] Isolates from collection of Timothy C. Paulitz. USDA ARS Pullman, WA

Table 2. Pythium root rot in seedlings of wheat (cv. Penawawa) treated with *P. fluorescens* strains and sown in soil infested with *P. ultimum*[a].

Treatment	Shoot height (cm)	Length of first true leaf (cm)	Shoot fresh weight (g)	Root length (cm)	Number of root tips	Average root diameter (mm)	Bacterial root colonization	
							TCB[b]	SCB[c]
Pf-5	11.1a[d]	6.0a	0.08a	38.2a	36.4a	0.47a	9.04a	7.79a
Q2-87	12.3a	6.8a	0.08a	56.1a	46.0a	0.38c	9.37a	8.01a
Q8r1-96	12.7a	6.4a	0.09a	41.1a	31.9a	0.39bc	9.14a	8.52a
F113	11.3a	5.1a	0.08a	42.5a	36.0a	0.46a	9.17a	8.46a
FFL3R49	10.6a	6.2a	0.09a	50.7a	39.7a	0.46a	9.45a	8.40a
2-79[e]	12.8a	6.4a	0.09a	54.0a	46.9a	0.43abc	9.24a	8.13a
Control +[f]	12.4a	7.0a	0.10a	41.4a	41.0a	0.44ab	9.31a	NA
Control –[g]	16.0	10.7	0.14	61.1	67.1	0.35	8.94a	NA
P. value	0.167	0.685	0.614	0.60	0.72	0.013	0.05	0.244

[a] Soils, with the exception of the negative control, were artificially infested with *Pythium ultimum* at a rate of 10^3 propagules/g of soil. The experiment was repeated four times, and a representative experiment is shown. The negative control was not included in the statistical analysis.

[b] Total culturable heterotrophic aerobic bacteria (log CFU/g of root) determined by the dilution end-point method with 1/10x TSB^+

[c] Seed coating bacteria. Population densities of bacterial strains (log CFU/g of root) used as a seed coating were determined by the PCR-based dilution end-point method with 1/3x KMB^{+++rif} and oligonucleotide primers B2BF and BPR4, and PCA2a and PCA3b for *phlD* and *phz* genes, respectively.

[d] Values are the means of five repetitions, with the exception of rhizosphere bacterial populations which are the means of six repetitions. Means with the same letter within each column are statistically similar according to Fisher's protected least significant difference test (P=0.05)

[e] Strain 2-79 produces PCA but not DAPG.

[f] Control positive. Inoculum of *P. ultimum* was introduced into this soil, as described above, and then non-bacterized wheat seed were sown.

[g] Control negative. Soil in this treatment was not infested with *P. ultimum* and non-bacterized wheat seeds were sown.

Figure 1. Inhibition of the mycelial growth of *Pythium* spp. by *P. fluorescens* strains. Values are the means of six repetitions. The experiment was repeated twice and results from a representative experiment are shown. Bars with the same letter within a *Pythium* spp. are not significantly different (P=0.05) according to Fisher's protected least significant difference test. Control - = negative control, Control + = positive control.

Figure 2. Root colonization by total aerobic heterotrophic bacteria (A), and strains 2-79, Q8r1-96, Z30-97 and Z34-97 (B) in the rhizosphere of wheat. Bacterial populations were determined in the rhizosphere two weeks after sowing and log transformed before data analysis. Values are the means of six replicates and are expressed as log of the mean (CFU/g of fresh weight of root). Bars with the same letter are not significantly different (P=0.05) according to Fisher's protected least significant difference. Control - = negative control, Control + = positive control.

Figure 3. Effect of *P. irregulare* on seedlings of wheat treated with different bacterial strains. Soils with the exception of the negative control were infested with *P. irregulare* at a rate of 10^3 propagules/g of soil. The experiment was repeated twice, and results from a representative experiment are shown. Each treatment was replicated six times with a single pot serving as a replicate. Bars with the same letter within a measurement are not significantly different (P=0.05) according to Fisher's protected least significant difference test. Control - = negative control, Control + = positive control.

Figure 4. Effect of *P. irregulare* on the roots of seedlings of wheat treated with different bacterial strains. Soils with the exception of the negative control were infested with *P. irregulare* at a rate of 10^3 propagules/g of soil. The experiment was repeated twice, and results from a representative experiment are shown. Each treatment was replicated six times with a single pot serving as a replicate. Bars with the same letter within a measurement are not significantly different (P=0.05) according to Fisher's protected least significant difference test. Control - = negative control, Control + = positive control.

Figure 1.

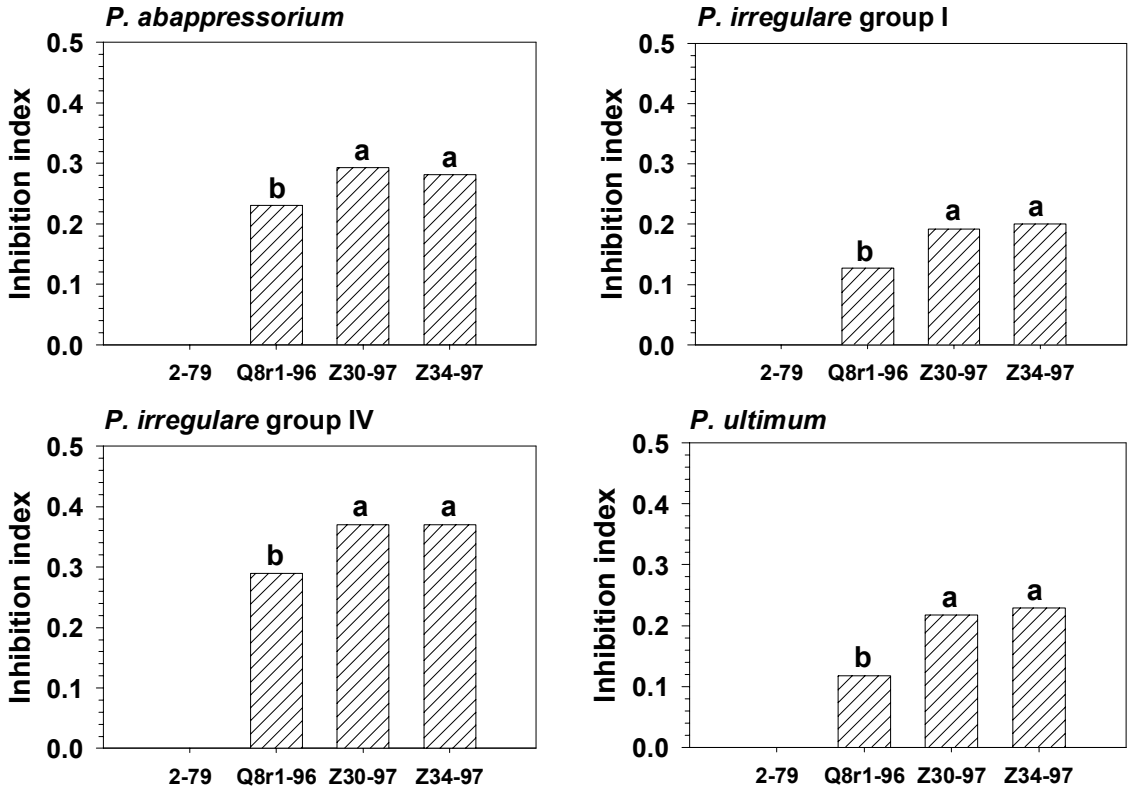

P. abappressorium
P. irregulare group I
P. irregulare group IV
P. ultimum
Inhibition index
0.0
0.1
0.2
0.3
0.4
0.5
a
b
2-79
Q8r1-96
Z30-97
Z34-97

Figure 2.

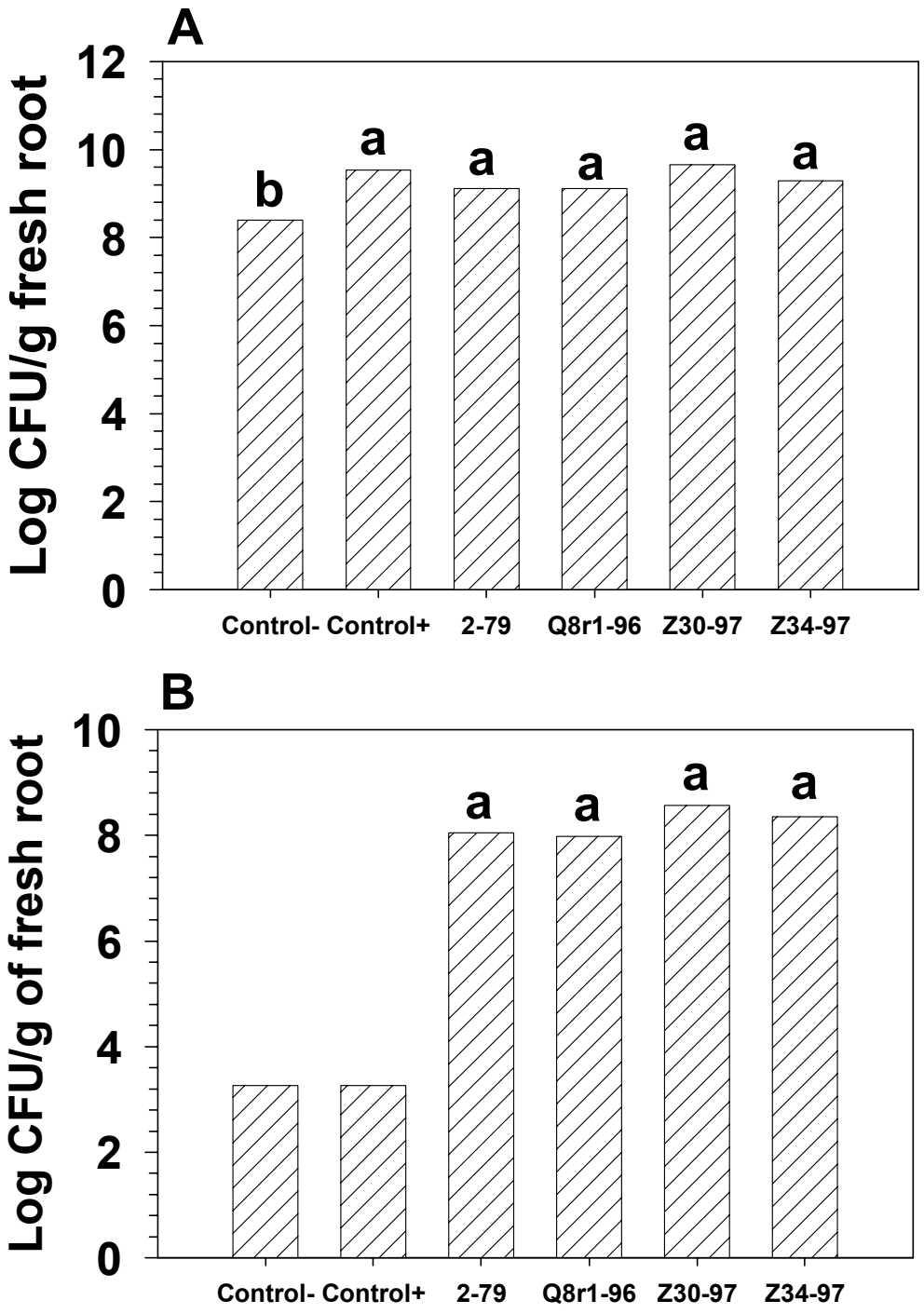

A
Log CFU/g fresh root
12
10
8
6
4
2
0
b
a
a
a
a
a
Control- Control+ 2-79 Q8r1-96 Z30-97 Z34-97
B
Log CFU/g of fresh root
10
8
6
4
2
0
a
a
a
a
Control- Control+ 2-79 Q8r1-96 Z30-97 Z34-97

Figure 3.

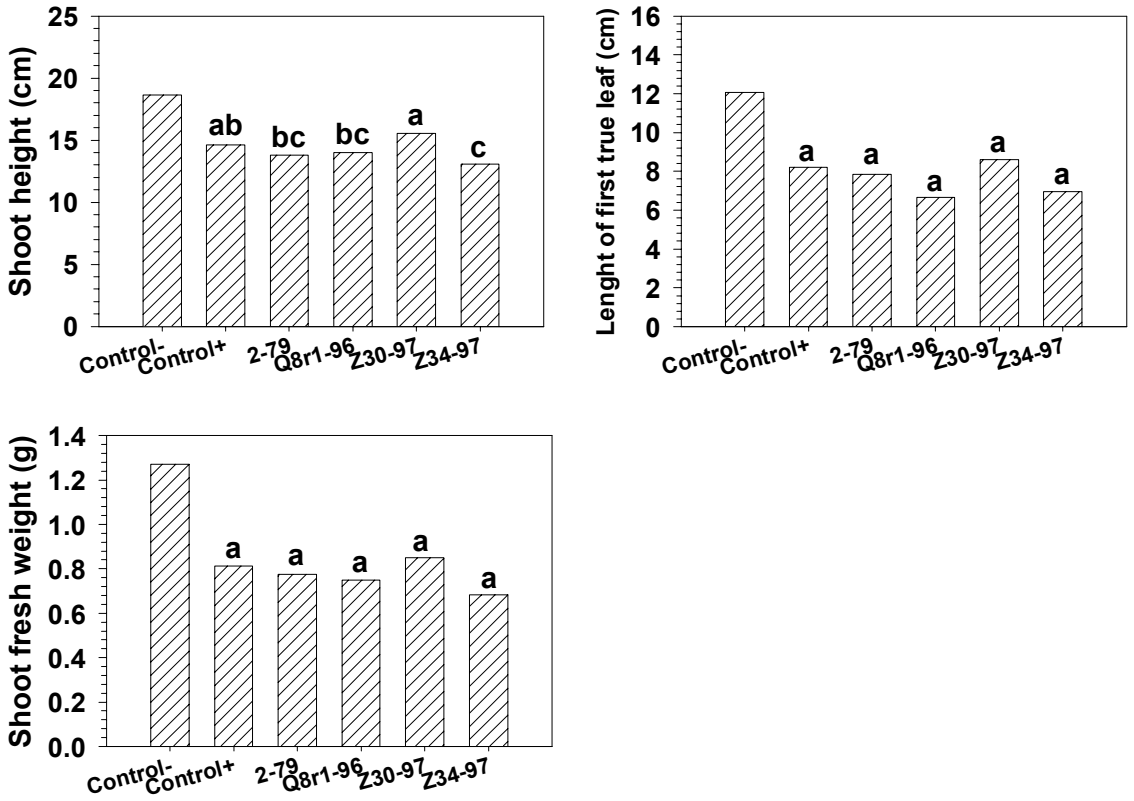
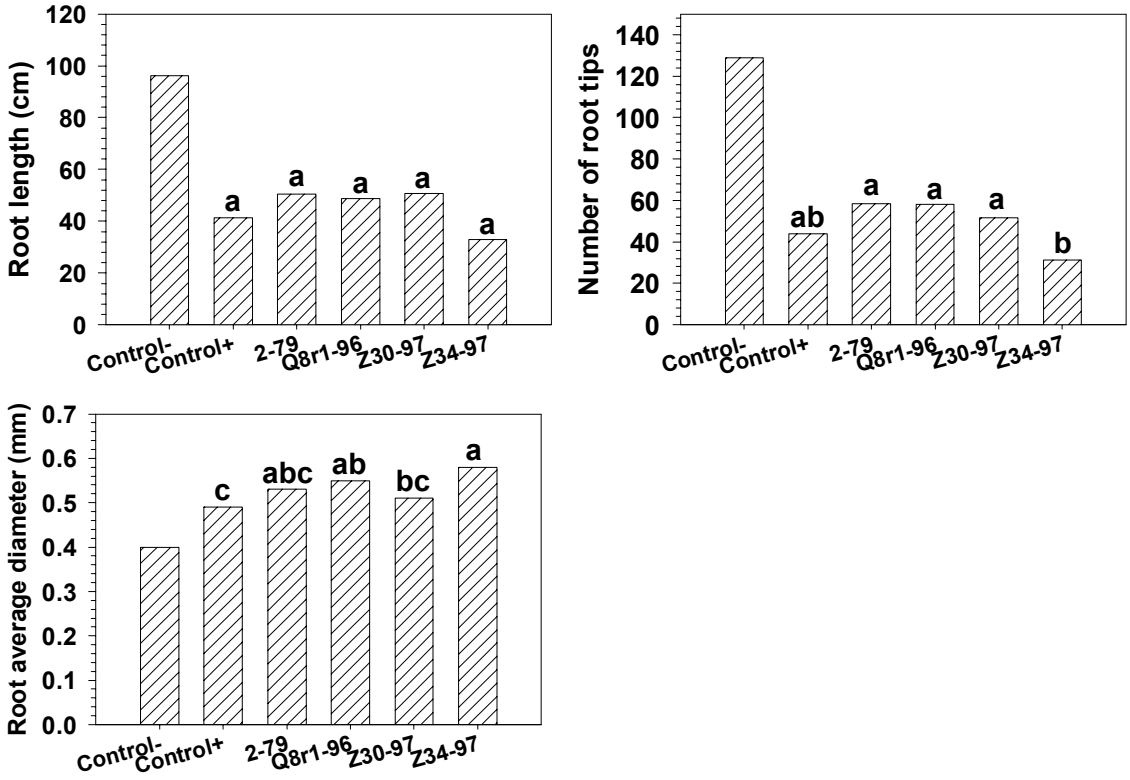

Shoot height (cm)
25
20
15
10
5
0
ab
bc
bc
a
c
Lenght of first true leaf (cm)
16
14
12
10
8
6
4
2
0
a
a
a
a
a
Shoot fresh weight (g)
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
a
a
a
a
a
Contrl-
Control+
2-79
Q8r1-96
Z30-97
Z34-97

Figure 4.


Root length (cm)
120
100
80
60
40
20
0
a
a
a
a
a
Control-
Control+
2-79
Q8r1-96
Z30-97
Z34-97
Number of root tips
140
120
100
80
60
40
20
0
ab
a
a
a
b
Control-
Control+
2-79
Q8r1-96
Z30-97
Z34-97
Root average diameter (mm)
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
c
abc
ab
bc
a
Control-
Control+
2-79
Q8r1-96
Z30-97
Z34-97

CONCLUSIONS

Population densities of indigenous DAPG-producing *P. fluorescens* were only found in the rhizosphere of wheat grown in soils with a history of wheat, flax or pea monoculture, even though some soil samples had been stored for over 22 years after being collected. In non-cropped soils or soils that had undergone crop rotation, DAPG producers were below the level of detection. Indigenous populations of DAPG producers enriched in a soil under continuous pea monoculture were able to suppress take-all of wheat. The role of DAPG-producing *P. fluorescens* in disease suppression was demonstrated by transferring the suppressive factor into a conducive soil. Furthermore, the microbial nature of the suppressiveness was confirmed by eliminating the microbial populations and disease suppression through soil pasteurization.

Indigenous populations of DAPG-producing *P. fluorescens* from wheat fields in which TAD had occurred were not able to control Pythium root rot of wheat. *P. abappressorium*, *P. irregulare* group I, *P. irregulare* group IV, and *P. ultimum* caused similar Pythium root rot severity in wheat seedlings grown in soils with or without DAPG producers. Strains of DAPG-producing *P. fluorescens* belonging to different genotypes caused inhibition of mycelial growth of *Pythium* spp. isolated from wheat fields in eastern Washington. *In vitro* assays indicated that *Pythium* spp. were less sensitive to DAPG than the take-all pathogen, which could account for the lack of biocontrol by DAPG producers.

Strains of DAPG-producing *P. fluorescens* belonging to different genotypes as well as recombinant strains, able to produce DAPG and phenazines, were not able to

reduce *Pythium* root rot of wheat caused by *P. irregulare* grup I or *P. ultimum*. The rapid infection of wheat seedlings by the isolates of *Pythium* spp. used in the assays could explain the lack of biocontrol.