CHARACTERIZATION OF THE ECOLOGICAL AND PHYSIOLOGICAL BASIS OF SUPERIOR RHIZOSPHERE COLONIZATION BY 2,4-DIACETYLPHLOROGLUCINOL-PRODUCING FLUORESCENT *PSEUDOMONAS* GENOTYPES

By

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To the Faculty of Washington State University:

The members of the Committee appointed to examine the dissertation/thesis of LEONARDO DE LA FUENTE find it satisfactory and recommend that it be accepted.

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Chair

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Abstract

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Rhizosphere isolates of *Pseudomonas fluorescens* producing the antibiotic 2,4-diacetylphloroglucinol (2,4-DAPG) are effective biocontrol agents, and are responsible for the suppressiveness of some soils to soilborne pathogens. The diversity within a worldwide collection of 2,4-DAPG-producers was assessed by sequencing *phlD*, a key gene in the 2,4-DAPG biosynthetic locus. Phylogenetic analyses resulted in groupings similar to those previously identified by BOX-PCR (genotypes A-Q and T). Exploiting polymorphisms in the sequence of *phlD*, allele-specific PCR primers were designed for the detection of strains of six genotypes. The primers allowed quantification of populations of strains competing in the rhizosphere. Soil was inoculated with all possible

combinations of *P. fluorescens* Q8r1-96 (genotype D), F113 (genotype K) and MVP1-4 (genotype P) to study competitive interactions among the strains. The three strains were equally effective at colonizing the wheat and pea rhizospheres when inoculated alone into the soil, but when introduced together the outcome of the competition varied depending on the host crop. Strains F113 and Q8r1-96 were more competitive than MVP1-4 on wheat; but MVP1-4 was the most aggressive competitor on pea. Alfalfa, bean, barley, flax, lentil, lupine, oat, pea and wheat differed quantitatively in the selection of indigenous populations of 2,4-DAPG-producers from the soil. Population densities of the inoculated strain Q8r1-96 differed significantly in the rhizosphere of eight pea cultivars; whereas MVP1-4 populations did not. Host crop plays a key role in modulation of the rhizosphere colonization by 2,4-DAPG-producers, especially in the competitiveness among strains occurring in the same soil. No correlation was found between the ability to utilize trehalose, benzoate or valerate as sole carbon sources and the rhizosphere competence of 2,4-DAPG-producers. A correlation was found between the use of these compounds by 2,4-DAPG-producers and the previous groupings identified by BOX-PCR. All strains grew similarly on seed or root exudates from wheat and pea. Differences in growth were detected only in wheat root exudates, where slow-growing strains included "premier" (Q8r1-96) and "average" (Q2-87) colonizer strains. The superior ability to colonize the rhizosphere of certain crops was not explained by the utilization of the carbon sources tested in our study.

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Dedication

To my family and friends in Uruguay.

GENERAL INTRODUCTION

Use of bacterial inoculants for plant-growth promotion: a historical framework

The use of bacterial inoculants in agriculture has been documented for over a century. Reports from late nineteenth century showed the beneficial effects of *Rhizobium* on legumes. In Russia, the use of *Bacillus* spp. and *Azotobacter chroococcum* on different crops was recommended by the government as early as 1930; and these inoculants as well as native *Pseudomonas* and *Beijerinckia* strains were used in India by the end of the 1960s (Kloepper et al., 1994). The first study in the USA on control of soilborne diseases by introduced microorganisms was performed by Hartley in 1921 (Cook, 1990). By introducing fungal and bacterial isolates, Hartley controlled damping-off caused by *Pythium debaryanum* in pine seedlings. Contemporary studies on the use of fluorescent *Pseudomonas* as inoculants to control soilborne diseases and promote plant growth were published in the late 1970s (Burr et al., 1978; Kloepper et al., 1980 a, b). For the last 25 years, fluorescent pseudomonads have been the focus of extensive research because of their ability to proliferate in the rhizosphere, produce a wide range of bioactive metabolites and inhibit or suppress a wide range of plant pathogens.

Fluorescent *Pseudomonas* as biocontrol agents

Strains of fluorescent pseudomonads with biocontrol activity have been isolated from agricultural soils from all continents (De La Fuente et al., 2004; Keel et al., 1996; Lee and Kim, 2001). The activity of inoculated strains against fungal, bacterial, oomycete and nematode plant pathogens and parasites has been extensively documented (Cronin 1997a, b; Haas and Défago 2005; Keel et al., 1992). Several mechanisms are involved in the protection of plants against phytopathogens by *Pseudomonas*: competition for nutrients such as carbon (Paulitz, 1991) or iron (Buysens et al., 1996; O'Sullivan and O'Gara, 1992); induced systemic resistance (van Loon

et al., 1998); and production of antibiotics such as phenazine derivatives, 2,4-diacetylphloroglucinol (2,4-DAPG), pyrrolnitrin, pyoluteorin, oomycin A, cyclic lipopetides and hydrogen cyanide (Haas and Défago, 2005; Raaijmakers et al., 2002; Thomashow and Weller 1995). Fluorescent *Pseudomonas* spp. strains producing the antibiotic 2,4-DAPG are the main focus of this work.

2,4-DAPG: a multifunctional metabolite

The locus for 2,4-DAPG biosynthesis was elucidated in 1999 (Bangera and Thomashow, 1999) and consists of six genes, *phlF* and *phlACBDE*. *phlD*, a key gene for the biosynthesis of 2,4-DAPG, encodes for a polyketide synthase with similarity to plant genes encoding chalcone/stilbene synthases (Bangera and Thomashow, 1999). PhlD plays a key role in the two proposed routes for the biosynthesis of 2,4-DAPG: synthesis of phloroglucinol from glucose (Achkar et al., 2005), and synthesis of the precursor monoacetylphloroglucinol (Bangera and Thomashow, 1999). The *phlD* gene is highly conserved in nature (Keel et al., 1996) and has been used extensively for molecular-based detection of bacteria producing 2,4-DAPG.

The purified polyketide 2,4-DAPG has broad antiviral, antibacterial, antifungal, antihelminthic and phytotoxic properties (Bangera and Thomashow, 1999; Keel et al., 1992). Recently, it was found to be active against vancomycin-resistant *Staphylococcus aureus*, an important human pathogen (Isnansetyo et al., 2003). The mechanism of action of 2,4-DAPG against phytopathogens is not fully elucidated, but it was reported to damage membranes and zoospores of *Pythium* spp. (de Souza et al., 2003a). In structure-function studies, purified 2,4-DAPG was an inhibitor of aldose reductase (AR) (Murata et al., 1992), an enzyme implicated in the metabolism of glucose to fructose. This enzyme is found in plants (Bartels et al., 1991), animals (de la fuente and Manzanaro, 2003) and fungi (Lee et al., 2003). In humans it is known

that a defective AR results in a cellular increase of the metabolic end products sorbitol and fructose, producing a disorder in the osmotic pressure in certain tissues. There are no studies relating this activity and the antibiotic nature of 2-4-DAPG toward plant pathogens. Besides the direct activity against pathogens (Keel et al., 1992; Ramette et al., 2001), the antibiotic also protects plants indirectly by inducing resistance in *Arabidopsis thaliana* against *Peronospora parasitica* (Iavicoli et al., 2003) and *Pseudomonas syringae* pv. *tomato* (Weller et al., 2004). 2,4-DAPG also affects root physiology directly by blocking amino acid uptake (Phillips et al., 2004).

2,4-DAPG is an autoinducer of its own synthesis and can function as a molecular signal (Schnider-Keel et al., 2000), influencing antibiotic expression in neighboring strains (Maurhofer et al., 2004). Complex microbial interactions in the rhizosphere also may potentially influence production of 2,4-DAPG, as illustrated by evidence that fungal metabolites can positively (Lutz et al., 2004; Siddiqui et al., 2004) or negatively (Duffy et al., 2004; Schnider-Keel et al., 2000) affect its biosynthesis.

Natural suppressive soils and 2,4-DAPG producers

Suppressive soils occur globally and are defined as "soils in which 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" (Baker and Cook, 1974). This natural phenomenon is the most effective way to control soilborne pathogens, which otherwise are difficult to manage with conventional methods such as fungicides or host resistance (Weller et al., 2002). Suppressive soils have been described for a broad group of soilborne pathogens, including *Gaeumannomyces graminis* var. *tritici* (Ggt), *Meloidogyne* spp., *Thielaviopsis basicola*, *Pythium ultimum*, *Rhizoctonia solani*, *Streptomyces scabies*, *Plasmodiophora brassicae*, and *Ralstonia solanacearum* (Weller et al., 2002). The best-

studied example of an induced suppressive soil is take-all decline (TAD), which occurs in wheat or barley against the disease take-all caused by Ggt (Weller et al., 2002). TAD is the spontaneous reduction in disease incidence and severity during wheat or barley monoculture following one or more severe outbreaks of the disease (Weller et al., 2002). The onset of TAD usually occurs after four to six years of monoculture and results from the stimulation and build-up of an antagonistic microflora. In fields identified in both Washington State (Raaijmakers and Weller, 2001) and The Netherlands (de Souza et al., 2003b), the build-up of fluorescent *Pseudomonas* producing the antibiotic 2,4-DAPG is responsible for TAD.

Diversity of 2,4-DAPG-producing *Pseudomonas*

Hundreds of 2,4-DAPG-producing isolates have been collected worldwide. Genetic diversity among these isolates has been studied by using different approaches: amplified ribosomal DNA restriction analysis (ARDRA) (Keel et al., 1996; McSpadden-Gardener et al., 2000; Picard et al., 2003), random amplified polymorphic DNA (RAPD) analysis (Mavrodi et al., 2001), whole-cell repetitive sequence-based-polymerase chain reaction with BOX primers (BOX-PCR) (Landa et al., 2002b; Mavrodi et al., 2001; McSpadden-Gardener et al., 2000), restriction fragment length polymorphism analysis of *phlD* (*phlD*-RFLP) (Landa et al., 2005; Mavrodi et al., 2001), sequence analysis of *phlD* (Mazzola et al., 2004; Ramette et al., 2001), and denaturing gradient gel electrophoresis (Bergsma-Vlami et al., 2005b). Early classifications defined three groups by ARDRA analysis (Keel et al., 1996; McSpadden-Gardener et al., 2000), and most of the strains corresponded to ARDRA group 2. Strains from ARDRA group 1 were separated from the other two groups by the ability to produce pyoluteorin, utilize certain carbon sources and deamidate the ethylene precursor 1-aminocyclopropane-1-carboxylate (McSpadden-Gardener et al., 2000; Wang et al., 2001). Strain F113 is the only known representative of ARDRA group 3.

Groups defined by BOX-PCR and *phlD*-RFLP correlated nearly perfectly (Mavrodi et al., 2001), and nowadays 22 different genotypes have been described (A-T, PfY and PfZ) by using either one of these two techniques (Landa et al., 2005; Mazzola et al., 2004; McSpadden-Gardener et al., 2005). Most genotypes produce similar quantities of 2,4-DAPG in vitro (Mavrodi et al., 2001) and in situ (Raaijmakers and Weller, 2001), have similar substrate utilization profiles (McSpadden-Gardener et al., 2000; Raaijmakers and Weller, 2001), and do not differ markedly when compared by classical bacteriological tests (McSpadden-Gardener et al., 2000; Raaijmakers and Weller, 2001). However, they differ significantly in their competitiveness in the rhizosphere, and at least on pea and wheat the genotype of an isolate is predictive of its ability to establish and maintain population densities sufficient to suppress plant pathogens (Landa et al., 2002b; Landa et al., 2003). Interestingly, the defined genotypes neither match the geographical nor the host crop origin of the bacterial isolates.

Rhizosphere colonization

Rhizosphere colonization is the process whereby bacteria, after being introduced into the soil, seeds or vegetatively propagated plant parts, can become distributed along the roots and survive for long periods of time in the presence of indigenous microflora (Weller et al., 1988). Rhizosphere competence is a highly desirable trait when it comes to the use of bacterial strains as inoculants in agricultural systems, since longevity in the rhizosphere is a key factor for a biocontrol agent to successfully protect the plant against soilborne pathogens. Different studies have focused on trying to elucidate which factors are involved in the colonization ability of rhizobacteria. These factors included cellular activities like motility, chemotaxis, prototrophy, ability to sequester iron and oxygen (Lugtenberg et al., 2001); and the ability to tolerate physical stresses including heat, desiccation and the presence of reactive oxygen species (Miller and

Wood, 1996; Sarniguet et al., 1997; Schnider-Keel et al., 2001). Genes involved in colonization include *sss* (phenotypic variation) (Sánchez-Contreras et al., 2002), *gacS*, *gacA*, *rpoS*, *algU* (global regulators) (Sánchez-Contreras et al., 2002; Sarniguet et al., 1997; Schnider-Keel et al., 2001), *dsbA* (periplasmic disulfide-bond forming protein) and *ptsP* (organic nitrogen utilization) (Mavrodi et al., 2004). Recent publication of the complete genome of the first biocontrol strain *P. fluorescens* Pf-5 (Paulsen et al., 2005) described the complexity of the metabolism of this bacterium. Many catabolic and transport capabilities to utilize plant-derived compounds were identified in the 7.1Mb genome (Paulsen et al., 2005), factors that could be involved in the rhizosphere competence of this strain. However, it has not been clearly demonstrated that the utilization of carbon and energy sources released from the plant roots plays an important role in rhizosphere colonization by introduced bacteria (Gu and Mazzola, 2001; Lugtenberg et al., 1999; Lugtenberg et al., 2001).

Quantification of 2,4-DAPG producers in natural soil.

Specific detection of inoculated bacterial strains in the soil is a difficult task due to the large background of indigenous microflora living in the environment. Moreover, the presence of bacteria genetically similar to the target strain requires a reliable tagging system. Spontaneous or introduced antibiotic resistance, or introduced genetic markers (*lux*, *lacZY*, among others) have been used in studies of rhizosphere colonization by introduced bacteria. For the detection of 2,4-DAPG producers, Raaijmakers et al. (1997) designed probes and primers targeting the *phlD* gene and via colony hybridization on selective media and PCR followed populations in the rhizosphere. McSpadden-Gardener et al. (2001) then developed a technique based on growth of dilution cultures on selective media and specific amplification of a *phlD* fragment by PCR. The PCR-based dilution-endpoint technique allowed a rapid processing of multiple samples, and the

possibility to determine the genotype of the target strain by *phlD*-RFLP. Nevertheless, this technique is suitable for single-bacteria inoculation, since it only detects the most abundant strain in a mixture. Landa et al. (2002a) compared these two techniques and the traditional dilution plating on selective media, and found that the three techniques are very similar and have comparable detection limits.

Superior colonization ability of 2-4-DAPG-producing genotypes

Characterization of populations of 2,4-DAPG-producing fluorescent *Pseudomonas* isolated from wheat roots grown in TAD soils obtained from Quincy (Washington, USA), revealed that one specific genotype represented 50% of the isolates (Raaijmakers and Weller, 2001). The same genotype was dominant on roots of wheat grown in other TAD soils obtained from Moses Lake and Lind (Washington, USA). D-genotype strains showed an outstanding ability to colonize wheat roots, and are exemplified by *Pseudomonas fluorescens* Q8r1-96 (Raaijmakers and Weller, 2001). A very low initial inoculum of Q8r1-96 (100 CFU g^{-1} soil) was enough to establish and maintain populations above log 5 CFU g^{-1} root on wheat during eight consecutive three-week cycles of growth in the greenhouse (Raaijmakers and Weller, 2001). In contrast, population densities of strains Q2-87 (B-genotype) and 1M1-96 (L-genotype) declined after the second cycle, were significantly lower than the populations of Q8r1-96, and were below the limit of detection by the eighth cycle (Raaijmakers and Weller, 2001). Different strains classified as the D-genotype were tested on wheat, and showed similar population dynamics as described for Q8r1-96 (Landa et al., 2003). PCR-based suppression subtractive hybridization (SSH) analysis, comparing strain Q8r1-96 ("premier" colonizer) against Q2-87 ("average" colonizer), detected several clones containing sequences unique to Q8r1-96 (Mavrodi et al., 2002). Most of the selected sequences contained open reading frames of unknown function, while others resembled

regulatory and colicin genes (Mavrodi et al., 2002). The role of these sequences in the rhizosphere competence of the D genotype is still under investigation. Further studies showed that the superior colonization ability described for genotype D on wheat is also found in pea, but in that case "premier" colonizer strains also belong to genotype P (Landa et al., 2002b).

Host specificity

Plant species have a marked effect on the genotypic and phenotypic characteristics of the community of associated microflora (Atkinson et al., 1975; Bergsma-Vlami et al., 2005a; Lemanceau et al., 1995; Marschner et al., 2004; Picard et al., 2003; Raaijmakers and Weller, 2001; Wieland et al., 2001). Moreover, plant species and cultivars respond differently to introduced and indigenous biocontrol agents (Landa et al., 2002b; Landa et al., 2003; Maurhofer et al., 1995; Mazzola et al., 2004; Okubara et al., 2003). Different degrees of specificity of recognition exist between the beneficial microflora and the host crop (Smith and Goodman, 1999). For 2,4-DAPG-producing fluorescent *Pseudomonas*, the degree of specificity of recognition with the host crop is not fully understood. Some evidence indicates that genotypes of this group of bacteria differ in their preference for host crops species (Bergsma-Vlami et al., 2005a; Landa et al. 2002b, 2003; Raaijmakers and Weller 2001) and cultivars (Mazzola et al., 2004; Okubara et al., 2003).

Objectives.

The general objective of this dissertation was to study ecological and physiological aspects involved in the rhizosphere colonization of different crops by BOX-PCR genotypes of 2,4-DAPG-producing *Pseudomonas*.

The specific objective of the first chapter was to develop a technique that allowed the detection of multiple BOX-PCR genotypes of 2,4-DAPG-producing fluorescent *Pseudomonas* competing in the rhizosphere. For this purpose, I sequenced a 700-bp fragment of the *phlD* gene from strains belonging to a worldwide collection of 2,4-DAPG-producers. The diversity among the strains was characterized by phylogenetical relationships based on *phlD* sequence data.

The objective of the second chapter was to study the effect of different plant species and cultivars on rhizosphere colonization by indigenous and introduced 2-4-DAPG producers. Two crops, wheat and pea, were selected to determine the effect of the host on competition among strains of different BOX-PCR genotypes.

The objective of the third chapter was to investigate the physiological basis for the superior ability of certain BOX-PCR genotypes to colonize the rhizosphere of specific crops. A collection of strains was screened for the utilization of specific compounds and seed or root exudates as sole carbon sources.

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Specific Contributions Of The Candidate And His Collaborators To The Present Studies

Chapter 1: Detection of genotypes of 2,4-diacetylphloroglucinol-producing *Pseudomonas* spp. by allele-specific PCR.

L. De La Fuente performed all experiments presented in this chapter. As stated in the manuscript, materials from a previous study by Dr. B. Landa were used for the detection of 2,4-DAPG-producing strains in soil and rhizosphere samples. Dr. D. Mavrodi, Dr. L. Thomashow and Dr. D. Weller contributed in the planning of the experimental design, discussion of results and preparation of the manuscript.

Chapter 2: Host crop affects rhizosphere colonization and competitiveness of 2,4-diacetylphloroglucinol-producing *Pseudomonas*.

Dr. B. Landa performed the greenhouse experiments for the selection of indigenous 2,4-DAPG producers by different crop species. The analysis of the dilution plates by PCR was performed by L. De La Fuente. Dr. B. Landa performed and analyzed the experiment of colonization of pea cultivars by indigenous populations of 2,4-DAPG producers. The rest of the experiments were performed by L. De La Fuente. Dr. B. Landa and Dr. D. Weller contributed in the planning of the experimental design, discussion of results and preparation of the manuscript.

Chapter 3: Utilization of trehalose, benzoate, valerate, seed or root exudates as sole carbon sources is not correlated with superior rhizosphere colonization by 2,4-diacetylphloroglucinol-producing *Pseudomonas* spp.

L. De La Fuente performed all experiments presented in this chapter. Dr. L. Thomashow and Dr. D. Weller contributed in the planning of the experimental design, discussion of results and preparation of the manuscript.

CHAPTER 1

Detection of genotypes of 2,4-diacetylphloroglucinol-producing *Pseudomonas* spp. by allele-specific PCR

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Abstract

Diversity within a worldwide collection of 2,4-diacetylphloroglucinol (2,4-DAPG)-producing fluorescent *Pseudomonas* spp. was assessed by sequencing the *phlD* gene. Phylogenetic analyses based on *phlD* sequences of seventy isolates supported the previous classification into eighteen rep-PCR genotypes (A-Q and T). Exploiting polymorphisms within the sequence of *phlD*, allele-specific PCR primers were designed and used with a PCR-based dilution endpoint assay to quantify the population sizes of the A-, B-, D-, K-, L- and P- genotype strains grown individually or in pairs in vitro and in the rhizosphere of wheat and bulk soil. Except for *P. fluorescens* Q8r1-96, which strongly inhibited the growth of *P. fluorescens* Q2-87, inhibition between pairs of strains grown in vitro did not affect the accuracy of the method. The allele-specific primer-based technique is a rapid method for studies of the interactions between genotypes of 2,4-DAPG producers in natural environments.

Introduction

Isolates of 2,4-diacetylphlorogucinol (2,4-DAPG)-producing fluorescent *Pseudomonas* spp. have been recovered from soil and the rhizosphere of crop species worldwide [1, 2, 3, 4, 5, 6], and from marine environments [7]. Many have been shown to be highly effective biocontrol agents of a wide variety of plant diseases: take-all, black root rot of tobacco, crown and root rot of tomato and beans, Pythium damping-off of cucumber and sugar beet, Phytophthora root rot of red pepper, cyst nematode and soft rot of potato [1, 3, 8, 9, 10, 11, 12, 13, 14, 15, 16]. Disease suppression by these strains is associated with the production of 2,4-DAPG, which has broad antiviral, antibacterial, antifungal, antihelminthic and phytotoxic properties [17] and is even active against drug-resistant clinical isolates of the human pathogen *Staphylococcus aureus* [7, 18]. In the rhizosphere, 2,4-DAPG may either directly inhibit soilborne pathogens [6, 10, 19, 20, 21, 22] or induce systemic resistance in the host plant [23]. 2,4-DAPG is an autoinducer of its own synthesis and can function as a molecular signal [24], influencing antibiotic expression in neighboring strains [25]. Complex microbial interactions in the rhizosphere also may potentially influence production, as illustrated by evidence that fungal metabolites can positively [26, 27] or negatively [24, 28] affect 2,4-DAPG synthesis.

Genetic diversity among 2,4-DAPG-producing isolates has been characterized by amplified ribosomal DNA restriction analysis (ARDRA) [2, 4, 29], random amplified polymorphic DNA (RAPD) analysis [10, 14, 30], whole-cell repetitive sequence-based-polymerase chain reaction (rep-PCR) [4, 31, 32], restriction fragment length polymorphism (RFLP) analysis of *phlD,* a key gene in the 2,4-DAPG biosynthetic operon [17, 30, 32, 33-35], sequence analysis of *phlD* [34, 36], and denaturing gradient gel electrophoresis (DGGE) [37].

Because it is so highly conserved, we use *phlD* as a genetic marker for producers of 2,4-DAPG. We have distinguished 18 *phlD* genotypes (A-Q and T) to date by rep-PCR within a worldwide collection of over 200 2,4-DAPG-producing (*phlD*$^+$) isolates [4, 31, 32], and these correlate closely with groupings revealed by RFLP analysis of *phlD* [30-33]. Additional genotypes were described recently by Mazzola et al. (PfY and PfZ) [36], and by McSpadden-Gardener et al. (R and S) [38]. Most genotypes produce similar quantities of 2,4-DAPG in vitro [30] and in situ [14], have similar substrate utilization profiles [4, 14], and do not differ markedly when compared by classical bacteriological tests [4, 14]. However, they differ significantly in their competitiveness in the rhizosphere, and at least on pea and wheat the genotype of an isolate is predictive of its ability to establish and maintain population densities sufficient to suppress plant pathogens [4, 31, 39].

Genes encoding resistance to foliar pathogens are abundant in most plant species, but resistance to many common and widespread soilborne pathogens is lacking. However, in a number of systems, plants have been shown to stimulate specific components of the soil microbial community that can possess antagonistic properties against plant pathogens [40-46]. One example of natural microbial defense of plant roots is take-all decline (TAD) [6], the suppression of take-all disease of wheat and barley caused by *Gaeumannomyces graminis* var. *tritici*. TAD has been reported in many locations around the world, and in some fields results from the build-up in soils of fluorescent *Pseudomonas* spp. that produce the antibiotic 2,4-DAPG [6, 10, 21]. 2,4-DAPG producers also play a key role in the suppressiveness of other soils [6], for example a soil from Switzerland suppressive to black root rot of tobacco caused by *Thielaviopsis basicola* [2, 6, 47].

The dynamics of the interaction and competition among 2,4-DAPG-producing genotypes in suppressive soils are poorly understood, because of limitations in the availability of suitable high throughput techniques to simultaneously detect and quantify multiple genotypes in soil and rhizosphere samples. Understanding these interactions is key to fully elucidating the contribution of each genotype to soil suppressiveness and explain why some genotypes are more abundant under specific conditions. Different techniques have been described for the quantification of total 2,4-DAPG populations in situ. Raaijmakers et al. [48] used colony hybridization with probes for sequences within *phlD* to detect and quantify indigenous populations of 2,4-DAPG producers in the wheat rhizosphere. Although this technique is accurate, it is labor intensive and does not discriminate among 2,4-DAPG genotypes. Later, McSpadden Gardener et al. [33] developed a quantitative dilution endpoint assay based on the terminal dilution in which *phlD* is detected by PCR after serially-diluted root washes are incubated in growth medium. This approach detects population sizes of *phlD*$^+$ fluorescent pseudomonads identical to those obtained by colony hybridization [49] but permits more rapid sample processing, and the dominant *phlD*$^+$ genotype in a sample can be identified by RFLP analysis of the amplified *phlD* fragments [30]. Landa et al. [31, 39] used the dilution-endpoint method to demonstrate that certain genotypes displace others when present in the same rhizosphere. However, a significant limitation of the assay is that in samples containing strains of two or more genotypes, a genotype comprising less than one-third of the total *phlD*$^+$ population (i. e., the subordinate strain) is difficult to detect by RFLP analysis [39].

The objectives of this study were to expand the ability of the PCR-based technique to quantify populations of 2,4-DAPG-producing fluorescent *Pseudomonas* spp. in the soil [33], and to develop a rapid and accurate approach to monitor the population dynamics of specific *phlD*$^+$

genotypes competing in the rhizosphere environment. Using the *phlD* sequences from strains of 18 rep-PCR genotypes, we first show that sequence analysis supported genotypes defined previously using molecular fingerprinting methods. The sequence information then was used to describe *phlD* allele-specific primers for A-, B-, D-, K-, L-, and P-genotype strains, which currently are among those most extensively studied [4, 31]. Finally, using these primers, we were able to monitor isolates of two competing genotypes in mixed cultures and in rhizosphere and soil samples even when one isolate was up to 1,000-fold less abundant than the other.

2. Materials and methods

2.1. Bacteria and culture conditions

The *phlD*$^+$ strains of fluorescent *Pseudomonas* spp. used in this study are shown in Table 1 and include representatives for most of the rep-PCR genotypes described to date [32, 39]. Except for *P. fluorescens* strain Phl1C2, provided by P. Lemanceau (Université de Bourgogne, Dijon, France), all of the strains were described previously. Genotypes were determined by using rep-PCR and/or RFLP analysis of *phlD* [4, 30-32]. Both approaches provide similar results [30]. *P. fluorescens* 2-79 produces phenazine-1-carboxylic acid [50] but not 2,4-DAPG. All strains were cultured at 28ºC on one-third-strength King's medium B agar (1/3x KMB) [51]. Stock cultures were stored in 1/3x KMB broth plus 20% glycerol at -80ºC. When appropriate, antibiotics were added to media at the following concentrations: kanamycin 25 μg ml^{-1} and gentamycin 12 μg ml^{-1}.

2.2. Amplification and sequencing of phlD *gene fragments*

The 37 2,4-DAPG-producing fluorescent *Pseudomonas* spp. strains from which *phlD* was sequenced were selected by considering as many different host crop and geographical origins as possible (Table 1), and included representatives of 18 genotypes determined by rep-PCR and *phlD*-RFLP analysis [4, 30-32]. A 745-bp *phlD* gene fragment from fluorescent *Pseudomonas* spp. strains was amplified by PCR using primers Phl2a and Phl2b [48]. The amplification products were gel-purified with a QIAEX II gel extraction kit (Qiagen, Valencia, CA) and sequenced by using an ABI PRISM BigDye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems, Foster City, CA) at the DNA Sequencing Laboratory (Washington State University, Pullmna, WA). Cycle sequencing was performed with primers B2BF and BPR4 [33] and Phl2a and Phl2b [48]. The *phlD* DNA sequences were assembled using the Lasergene package (DNA Star Inc., Madison, WI). Sequencing errors in the consensus sequences were detected using the translating BLAST function blastx [52] (National Center for Biotechnology Information, NCBI). GenBank accession numbers for the resulting *phlD* sequences are shown in Table 1.

2.3. Phylogenetic analysis

Thirty-three *phlD* sequences (Table 1) were identified in the non-redundant GenBank dataset (NCBI) by using the WWW-based NCBI nucleotide BLAST search engine [52] with default parameters and the 2,4-DAPG biosynthetic locus of *P. fluorescens* Q2-87 as the query sequence. Only sequences longer than 600 bp were considered for further analysis. These

sequences, together with the 37 *phlD* sequences obtained in this study (Table 1), were aligned using the ClustalW service at the European Bioinformatics Institute (URL http://www.ebi.ac.uk/clustalw). Sequences were trimmed to identical length of 599 bp. Phylogenetic analyses were performed with Phylip 3.62 [53] using the neighbor-joining method. For comparison purposes, similar analyses were performed with the maximum parsimony method. For both phylogeny inferring methods, DNA and protein sequences were used. Confidence of the topology of the neighbor-joining phylogenetic tree for the *phlD* gene was performed by bootstrap analysis on 1,000 resamplings. Branches with bootstrap values below 50% were collapsed. The phylogenetic tree was visualized and edited by using TreeView (Win32) (URL http://taxonomy.zoology.gla.ac.uk/rod/rod.html).

2.4. Design of phlD *allele-specific primers*

A subset of partial *phlD* sequences was selected from GenBank including sequences from eight strains representative of five genotypes: *P. fluorescens* UP61 (genotype A), CHA0 (genotype A), Pf-5 (genotype A), Q8r1-96 (genotype D), 11-18 (genotype D), F113 (genotype K), 1M1-96 (genotype L), and PILH1 (genotype M). These eight sequences and those obtained in this study corresponding to 7MA12 (genotype O), MVP-1-4 (genotype P), MVW1-1 (genotype P), and MVW4-2 (genotype Q) were aligned by using ClustalW and utilized to design allele-specific PCR primers (Table 3) with the aid of OLIGO V.6.65 software (Molecular Biology Insights Inc., Cascade, CO) according to the following criteria: i) oligonucleotide length of 18-25 bp; ii) melting temperature (T_m) of $\geq$ 55°C with differences $\leq$13.5°C between primer pairs; iii) 5 to 7 nucleotides at the 3' end include at least 1 allele-specific nucleotide; iv) 3'

terminal nucleotide is different from A; v) no predicted hairpin loops, duplexes or primer-dimers; and vi) amplification product ≥ 300bp. An in silico test of primer specificity was conducted by running the primer sequences against the non-redundant GenBank dataset with parameters set for the identification of short nearly exact matches.

2. 5. Allele-specific PCR

Conditions for allele-specific amplification of *phlD* initially were determined with purified bacterial genomic DNA (50 ng per reaction approximately) from representative strains of the A, B, D, K, L and P genotypes utilized as templates. PCR reactions contained 1x *Taq* reaction buffer (Promega Corp., Madison, WI), 1.5 mM $MgCl_2$, 200 µM of each dATP, dCTP, dGTP, dTTP (Perkin-Elmer, Norwalk, CT), 20 pmol of each primer and 1.2 units of *Taq* DNA polymerase (Promega). Amplification was performed with a PTC-200 thermal cycler (MJ Research, Watertown, MA). The cycling program consisted of initial melting at 95°C for 180 s followed by 35 cycles of melting at 94°C for 60 s, annealing at 60°C for 60 s, and extension at 72°C for 60 s; and a final extension at 72°C for 300 s. The annealing temperature, and $MgCl_2$ and DMSO concentrations subsequently were adjusted as shown in Table 3 to optimize the reaction for each genotype, and specificity was evaluated with template DNA from one-day-old single colonies of 70 strains (Table 1) including representatives of 18 genotypes. Freeze-thawed cell suspensions (2.5µl) were used as templates for PCR reactions when samples from serial dilution plates or broth cultures were analyzed. PCR amplification products were separated in 0.8% agarose gels containing ethidium bromide (0.5 µg/ml).

2.6. Genotype detection by allele-specific PCR in mixed cultures

Strains were tested individually and in pairwise combinations by using a protocol similar to that of Landa et al. [39] in order to determine the detection limit of each genotype by *phlD*-directed allele-specific PCR. Analyses included *P. fluorescens* Pf-5, Q2-87, Q8r1-96, F113, 1M1-96, and MVP1-4 alone; Q8r1-96 plus Pf-5, Q2-87, F113, 1M1-96 or MVP1-4; and F113 plus MVP1-4. Overnight cultures of each strain were harvested by centrifugation in a microfuge (10,000 x g, 5 min), suspended in 1/3x KMB to an absorbance at 600nm (OD_{600nm}) of 0.1 (approximately $1x10^8$ CFU ml^{-1}) by using a SAFIRE microplate reader (Tecan Systems, Inc., San Jose, CA), and added (250 µl per well) to 96-well microtiter plates. For pairs of strains, 125 µl of appropriate tenfold serial dilutions were added to the wells to obtain mixtures ratios of 1:1, 1:10, 10:1, 1:100, and 100:1. Samples were replicated three times and the entire experiment was conducted twice. The plates were incubated at room temperature for 72 h and bacterial growth was monitored at 24-h intervals by measuring the OD_{600nm}. Samples (20 µl) were taken at 0, 24, 48 and 72 h from each well, frozen at -20°C and thawed preceding analysis by PCR with primers specific for each genotype in the culture.

2.7. Inhibition between genotypes in vitro

In vitro assays were performed in order to determine whether the inability to detect certain genotypes by PCR when they are grown in combination resulted from inhibition of one strain by another or was caused by ineffective PCR amplification by the allele-specific primers. Derivatives of strains Q2-87, Q8r1-96 and MVP1-4 marked with mini-Tn*7*gfp1 or mini-Tn*7*gfp2

[54], which confer resistance to kanamycin (Q2-87Km, MVP1-4Km) or gentamicin (Q8r1-96Gm), respectively [55], were used, allowing differentiation of viable cells of the marked strains by plating on selective media containing the corresponding antibiotics. Strains were paired on the basis of an expectation of significant growth inhibition (Q8r1-96Gm and Q2-87Km) or little growth inhibition (Q8r1-96Gm and MVP1-4Km). Cultures (4 ml) of individual strains or mixtures (Q8r1-96Gm plus Q2-87Km or MVP1-4Km) at ratios of 1:1, 1:10, and 10:1 in 1/3x KMB broth were incubated on a rotary shaker (200 rpm) at 28°C for 72 h and viable cell numbers were scored at 24-h intervals by plating on 1/3x KMB agar amended with either kanamycin or gentamicin.

2.8. Genotype detection by allele-specific PCR in soil and rhizosphere samples

Materials from a previous study by Landa et al. [39] were employed to test the ability of genotype-specific primers to detect 2,4-DAPG producers in soil and on roots. Landa et al. [39] previously evaluated the ability of *P. fluorescens* strains Q8r1-96, F113 and Pf-5 to compete in the rhizosphere of wheat and in bulk soil. A Shano silt loam was inoculated with each strain or with 1:1 mixtures of Q8r1-96 and F113 or Q8r1-96 and Pf-5 (each at approximately log 4 CFU g^{-1} soil). Three treatments were established. The first consisted of pots filled with natural non cropped soil inoculated with the bacteria and sown with wheat (*Triticum aestivum* L.) seeds (cv. Penawawa). After three weeks (one "cycle"), the plants were harvested, roots with adhering rhizosphere soil (designated RRS) were assayed for the introduced bacteria, and the soil and remaining roots were returned to the pots and sown again to begin the next cycle. Wheat was grown for a total of five 3-week cycles and the roots were assayed for the introduced bacteria

after each cycle (see below). A second treatment consisted of pots filled with soil inoculated with the bacteria, sown to wheat and cycled five times. Unlike the first treatment, however, the roots were not incorporated back into the soil at the end of each cycle, and the soil without roots (designated non-rhizosphere soil, NRS) was assayed for the introduced bacteria. The third treatment consisted of pots filled with inoculated soil and watered weekly, but in which no plants were sown. At 3-week intervals the soil was removed, shaken, returned to the pots, and samples (designated bulk soil, BS) were processed as described previously [49]. Densities of Q8r1-96, F113 and Pf-5 in all three sets of samples were determined by using the PCR-based dilution endpoint assay [33] followed by RFLP analysis of the PCR amplification products from terminal dilution cultures (TDC) of each treatment. As reported by Landa et al. [39], amplification products from treatments in which strains Q8r1-96 or F113 had been introduced individually into the soil produced the expected *phlD*-RFLP profiles. However, mixed treatments inoculated with both Q8r1-96 and F113 revealed only the profile characteristic of F113. We used the allele-specific primers to assay dilutions of the cultures that had been recovered from root and soil samples and stored at -20°C for over 18 months, and compared the results to those obtained previously [39].

2.9. Data analysis

Population data from the soil studies were converted to log CFU (colony-forming units) per gram of soil or root fresh weight to satisfy the assumptions of analysis of variance (ANOVA). The colonization of the rhizosphere or soil by each strain introduced individually or as a mixture was determined by two methods. Area under the colonization progress curve

(AUCPC) [31] was calculated by using the trapezoidal integration method [56]. Mean colonization was calculated as the mean population density across all cycles [31]. Differences in population densities among treatments were determined by ANOVA and mean comparisons among treatments were performed by Fischer's protected least significance difference (LSD) test at $P = 0.05$ by using the STATISTIX 8.0 software (Analytical Software, St. Paul, MN). For soil or rhizosphere samples in which no PCR signal was detected, population density was set at the detection limit of the allele-specific PCR assay (log 3.26 CFU g^{-1} of sample).

3. Results

3.1. phlD *sequences and phylogenetic analyses*

With the 37 *phlD* gene sequences obtained in this study, we completed the sequencing of representatives of 18 of the 20 rep-PCR genotypes (A-Q and T) described to date. Whenever available, *phlD* sequences from at least three strains from each genotype were selected from GenBank and our data (Table 1) for phylogenetic analyses. However, for some of the genotypes (G, H, I, K, and N), sequences from only a single strain were available. Comparison of sequences from 18 rep-PCR genotypes and 2 newly described genotypes [36] confirmed previous observations [2, 33, 34] that *phlD* is highly conserved among 2,4-DAPG-producing fluorescent *Pseudomonas* spp. A neighbor-joining tree (Fig. 1) comprising *phlD* sequences from 70 fluorescent *Pseudomona*s spp. strains, shows that the previous classifications based on rep-PCR and *phlD*-RFLP [4, 30-32] have a good correlation with the clusters obtained with 599 bp of *phlD* gene sequences. The same tree topology was obtained by inferring the phylogeny with the

neighbor-joining method on protein sequence data and with maximum parsimony on both DNA and protein sequence data.

3.2. Allele-specific primer design

A subset of thirteen *phlD* sequences from strains of rep-PCR genotypes A, B, D, K, L, M, O, P and Q were aligned to detect differences that could be exploited to design allele-specific primers. Despite the overall similarity among sequences, which ranged in identity from 76.0 to 99.7%, sufficient differences were identified to generate primer sequences that had 100% identity only with the targeted *phlD* alleles from strains of genotypes A, B, D, K, L, and P (data not shown). Table 2 shows the primer sequences, melting temperatures, and positions within *phlD*. The primer pairs and amplification conditions for detecting each genotype are shown in Table 3.

The six pairs of allele-specific primers amplified *phlD* in all representatives of the target genotypes among the 70 strains (Table 1) screened. The amplification products from nine well-described strains representing the six genotypes that are the focus of this study are shown in Figure 2. Two instances of nonspecific amplification also were observed: genotype K-specific primers also amplified *phlD* in strains of genotypes C and M, and genotype L-specific primers amplified *phlD* in strains of genotype Q (data not shown).

3.3 Sensitivity of detection in vitro

Strains of genotypes K (F113) and P (MVP1-4) were detected by their respective primers at 24, 48, and 72 h whether grown individually or mixed together and regardless of the ratio at

which they were inoculated (Table 4). Similarly, strains of genotypes A (Pf-5), B (Q2-87), K, L (1M1-96) and P were detected at 24, 48 and 72 h whether grown individually or after co-inoculation with strain Q8r1-96 at ratios of 1:1 or 10:1 (Table 4). The detection limit in such mixtures was comparable to that when the strains were grown individually, or approximately log 5.7 cells ml^{-1} for the B-, D-, K- and P- genotype strains and about 10-fold higher for A- and L-genotype strains (Table 4). The OD_{600nm} values for cultures of individual strains and strain mixtures did not differ significantly over the course of the 72-h experiment (data not shown).

When Q8r1-96 was inoculated at a ratio of 10:1 with other strains, both it and strains of the A, K and P genotypes were detected at 24, 48 and 72 h. In contrast, the B-genotype strain Q2-87 was detected only at t=0 and the L-genotype strain 1M1-96 was not detected at any sampling time (Table 4). When Q8r1-96 was introduced in 100-fold excess, neither Q2-87 nor 1M1-96 were detected at any time. However, at a ratio 1:100 (Q8r1-96 to any other strain), Q8r1-96 always was detected (Table 4).

3.4. Competition in broth cultures

P. fluorescens Q8r1-96Gm inhibited the growth of Q2-87Km (B genotype) at ratios of 1:1 and 10:1. Plating on antibiotic-containing media revealed that when Q8r1-96Gm was the more abundant strain in the inoculum (ratio of 10:1) (Fig. 3), the density of Q2-87Km after 24 h was nearly 9 orders of magnitude less than when it was cultured alone. However, when the strains were inoculated at a ratio of 1:1, the populations of Q2-87Km decreased by only log 2-3 CFU ml^{-1}. No significant inhibition occurred when Q2-87Km was the more abundant component of the inoculum (Q8r1-96Gm and Q2-87Km ratio of 1:10). Given that the threshold density for

the detection of these strains by PCR is log 5.7 CFU ml^{-1}, it appears that when Q8r1-96 is introduced at a density greater than that of Q2-87, the density of Q2-87 drops below the threshold for detection by PCR (Table 4). Results were consistent when the experiment was repeated.

A similar but less severe inhibition of MVP1-4Km (P genotype) by Q8r1-96Gm was observed when these two strains were grown together. The effect was the most pronounced when the ratio of Q8r1-96Gm to MVP1-4Km was 10:1, which caused the density of MVP1-4Km to decrease by about log 4 CFU ml^{-1} as compared to its population when grown alone. The population density of MVP1-4Km stabilized close to log 6 CFU ml^{-1}, above the threshold of log 5.7 CFU ml^{-1} required for detection by PCR in mixed cultures and explaining why it could be detected in the presence of Q8r1-96 (Table 4).

3.5. Detection of 2,4-DAPG-producing Pseudomonas *spp. in the soil and rhizosphere*

The analysis of samples from a previous study by Landa et al. [39] was performed by PCR with primers specific for the D- and K- genotypes. First we determined whether these primers were as efficient as B2BF and BPR4, the primers employed in the standard dilution endpoint assay [33]. Population densities of F113, introduced alone or in combination with Q8r1-96 and detected with the K-genotype primers in the rhizosphere (RRS, Fig. 4D), bulk soil subjected to wheat cropping (NRS, Fig. 4E) and bulk soil without cropping (BS, Fig. 4F), were identical to those reported originally by Landa et al. [39]. Similarly, the populations densities of Q8r1-96 introduced alone into the soil and detected with the D-genotype specific primers in the RRS, NRS, and BS samples (Fig. 4A, B and C) were identical to those reported earlier [39]. The

detection limit with the D and K genotype specific primers was log 3.26 CFU g^{-1} of sample, equivalent to that for the generic *phlD*-primers B2BF and BPR4 [33, 49].

Next we determined whether the D-genotype-specific primers could detect Q8r1-96 when it was not the dominant genotype present in a mixture. In mixed treatments in which F113 was the dominant strain, Landa et al. [39] could not detect Q8r1-96; however we consistently detected Q8r1-96 in the RRS, NRS, and BS samples (Fig. 4A, B, and C). In samples from all three treatments, the population density of Q8r1-96 was significantly reduced by about 10- to 1,000-fold in the presence of F113. Furthermore, the AUCPC for Q8r1-96 introduced in combination with F113 was significantly smaller ($P < 0.05$) than when it was introduced alone (Table 5). The negative effects of F113 on the density of Q8r1-96 were greater in RRS samples than in NRS and BS samples (Fig. 4). In contrast, population densities of F113 were not affected by the presence of Q8r1-96 (Fig. 4A, B, and C, Table 5) in samples from any of the three treatments, and were identical to those reported by Landa et al. [39].

Finally, we analyzed samples from the earlier study [39] in which the A-genotype strain Pf-5 was introduced alone or in combination with Q8r1-96. Population densities of Pf-5 introduced alone and detected with the genotype A-specific primers in RRS, NRS and BS samples were identical to those reported previously [39]. However, when introduced with Q8r1-96, both strains were readily detected in the soil prior to cycling ("cycle 0") (data not shown) but only Q8r1-96 was detected in RRS, NRS and BS samples thereafter, indicating that populations of Pf-5 quickly dropped below the detection limit of log 3.26 CFU g^{-1} of sample. Population densities of Q8r1-96 detected with the D genotype specific primers in the presence or absence of Pf-5 were identical to those reported earlier [39] (data not shown).

4. Discussion

Based on the allele-specific PCR primers for six of the most studied 2,4-DAPG-producing genotypes, we developed an effective method for tracking populations of *phlD*$^+$ strains in the soil without the need for introducing genetic markers into the bacteria. Using these primers we improved the technique developed by McSpadden-Gardener et al. [33] by making it suitable for fine-scale studies of interactions among 2,4-DAPG producers in the rhizosphere, while retaining its high-throughput capabilities.

Ecologically-based pest management [57] requires a detailed understanding of the interactions between pathogenic and beneficial microorganisms and their hosts, as well as the impact of environmental conditions on that complex system. Historically, detection of both beneficial and pathogenic microorganisms in the rhizosphere or bulk soil relied on dilution plating and/or time-consuming biochemical and immunological techniques [58]. However, with the development of DNA-based techniques including numerous forms of PCR [57, 59], microbial ecologists have greatly expanded their understanding of interactions among culturable and non-culturable microorganisms in soil. We continue to focus our attention on interactions among *phlD*$^+$ fluorescent *Pseudomonas* spp. because of the key role that these beneficial bacteria play in the defense of roots against soilborne pathogens.

With the 37 strains sequenced in this study (Table 1), a comprehensive collection of *phlD* sequences obtained from 70 2,4-DAPG producers isolated worldwide is now available in GenBank. The clusters identified with our phylogenetic tree (Fig. 1) did not match with the host crop where strains were isolated or with their geographical origin (Table 1). Most importantly, however, is the finding that the clusters correspond to the rep-PCR genotype classification previously described by our laboratory [4, 30-32]. Therefore it is reasonable to assume that

strains not previously identified as to genotype by rep-PCR, but found in the same cluster with well-defined strains, can be considered to belong to the same genotype. For example, strains 11-18, 37-27 42-27, 22-27, 7-37, 6-28, 18-33, 39-8, 42-36, 19-41 and 3-1 [60] most probably belong to the genotype D group (Fig. 1). This conclusion is further supported by the fact that strains 11-18 and 37-27 were confirmed to be D genotype by *phlD*-RFLP and allele-specific PCR (Table 1). These findings represent the first report of D-genotype isolates occurring in Europe. Previously, D-genotype strains had been isolated only from soils collected in the USA. On the other hand, strains 19-30 and 19-7 from that study [60] appear to define an independent branch of the tree (Fig. 1), not clustering with any previously described genotype and most probably representing a new one. In addition, according to the neighbor-joining tree topology, strain PITR2 may belong to the M genotype (Fig. 1). This tree, built from *phlD* sequences of 70 fluorescent *Pseudomonas* spp. strains, establishes the sequence of *phlD* as a valid new criterion for classifying 2,4-DAPG producers according to the genotypes defined in our laboratory [4, 30, 31]. This is especially important since it was previously shown that the genotype is predictive of the colonization ability of the strains on at least some host plants [31, 39].

We exploited naturally-occurring variation within the sequence of *phlD* to develop allele-specific PCR primers for rapid and specific detection of certain genotypes of 2,4-DAPG-producing *Pseudomonas* spp. By aligning *phlD* sequences deposited in GenBank with other sequences obtained in our laboratory, we were able to detect differences that allowed us to design a set of oligonucleotides that would discriminate among A-, B-, D-, K-, L-, and P-genotype strains. We initially tested the primers with purified bacterial DNA in order to adjust the reaction conditions for maximal specificity and yield. The addition of DMSO helped to increase the yield of amplified fragments in some reactions, and reducing the concentration of

$MgCl_2$ significantly improved the specificity of amplification with the K-gen and L1-exten primers (Table 3).

The specificity of the allele-specific primers was then evaluated on a set of strains representing 18 known rep-PCR genotypes. Results of this screening indicated that with few exceptions, the primers gave highly specific amplification. Among the exceptions were the K-genotype primers, which also detected C- and M-genotype strains, and the L-genotype primer, which amplified *phlD* from Q-genotype strains. Both a perfect match at 3' end of the primer and the nature of the terminal base are critical for the specificity of *Taq* polymerase-based amplification [61]. According to Ayyadavera et al. [62], placement of an A at the 3' end of the oligonucleotide often leads to the lowest possible degree of discrimination in allele-specific PCR. Unfortunately, it was necessary to design both the K- and the L-genotype primers with A in the terminal position because of the absence of polymorphic sites within *phlD* that would have enabled us to design primers that would distinguish K- and L-genotype strains from strains of the C- , M-, and Q-genotypes.

The detection limit for the direct amplification with allele-specific primers when strains were grown in vitro was approximately log 5.7 CFU ml^{-1} for strains of genotypes B, D, K, and P, and log 6.7 CFU ml^{-1} for genotype-A and -L strains. It is difficult to assess exactly how much template DNA corresponds to these large numbers of cells because templates were prepared from freeze-thawed cultures in which only a fraction of the total bacterial population releases DNA. The physiological state of the cells would influence the limits of detection by PCR, as was previously demonstrated [63]. That could also be an explanation for our high detection limit, since the bacterial cultures used in our study were in the stationary phase of growth when the PCR was performed.

Landa et al. [49] found that the detection limit for rhizosphere samples by the dilution endpoint assay was log 3.26 CFU g^{-1} in soil and fresh root samples and we found the same detection limit with the allele-specific primers, indicating that the allele-specific method is as sensitive as the original technique [33]. The detection limit for soil and rhizosphere samples also depends on the release of template DNA from cells that have been frozen and thawed during the PCR-based dilution endpoint assay. For our purposes, the speed and robustness of the assay outweigh the drawback that the detection limit is greater than 1,000 cells g^{-1} of sample.

The most interesting application of allele-specific PCR is the detection and quantification of two (this study) or more (De La Fuente, L., Landa, B.B. and Weller, D.M., unpublished results) strains of different genotypes of 2,4-DAPG-producers competing in soil or on roots. Allele-specific PCR has a clear advantage over the well-tested dilution endpoint assay [33], which is limited by its inability to detect less abundant genotypes in soil or root samples when their densities are 3- to 6-fold less than the dominant genotype [39]. This is due to the generic characteristic of the primers (B2BF/BPR4) used in the technique, which amplify all rep-PCR genotypes A to T [32, 33, 38] and PfY and PfZ [36]. Using samples from a previous study [39], we demonstrated the utility of the technique by quantifying the density of Q8r1-96 in competition with F113 in three different types of rhizosphere and soil samples (RRS, NRS and BS, Fig. 4 and Table 5). Allele-specific PCR is ideal for tracking 2,4-DAPG producers introduced into the soil, but more importantly it allows fine scale studies of interactions and competition among genotypes indigenous to disease suppressive and crop monoculture soils without the need for introduced genetic markers. Studies to date [6, 10] suggest that these soils contain multiple genotypes, with one genotype often dominant. However, how interactions among the genotypes contribute to pathogen or competing genotype suppression, and whether

strains of different genotypes inhabit different niches in the soil and rhizosphere environment are questions that have been difficult to address with previously available techniques.

Given the overall similarity among genotypes of 2,4-DAPG producers [4, 14, 30], we were surprised to detect significant inhibition in vitro of the B-genotype strain Q2-87 and the L-genotype strain 1M1-96 by the D-genotype strain Q8r1-96. For example, when Q8r1-96 and Q2-87 were combined in a ratio of 10:1, the density of Q2-87 dropped precipitously from log 6 to log 2 CFU ml^{-1} after 24 h (Fig. 3), below the detection limit for the PCR assay. We tried to bypass the growth step in the assay (in which dilutions of root washes are transferred into 1/3x KMB) and detect the bacteria directly in root washes to limit the effect of inhibition of one genotype on another. However, this approach increased the detection limit several-fold and the PCR reactions often failed, probably because of low concentration of target cells and the presence of inhibitors from the soil [57].

The inhibition of Q2-87 by Q8r1-96 detected in vitro also occurs in the wheat rhizosphere. With the use of de Wit replacement series, Landa et al. [39] demonstrated a competitive disadvantage for strain Q2-87 or strong antagonism by Q8r1-96 against Q2-87 in the wheat rhizosphere. Work in our laboratory [55] has shown that Q8r1-96 produces bacteriocin-like inhibitors active against Q2-87. Bacteriocin activity also was detected in strains of several other genotypes. Theoretical models of interactions between bacteriocin-producing and -sensitive strains have shown that inhibition depends on the relative strain concentrations [64]. If both are present at equal densities the bacteriocin-producing strain cannot prevail, but when it is the more abundant strain, it will antagonize the sensitive strain. This model is consistent with the observed patterns of inhibition of Q2-87 by strain Q8r1-96. More important is the impact of inhibition on the allele-specific and dilution endpoint PCR assays. However, our results suggest that these

assays may be compromised only when inhibition of one genotype by another is extremely severe, as in mixtures of the D- and B- genotype strains. In such cases the density of the antagonized strain would be underestimated. During mild inhibition, as in mixed cultures of Q8r1-96 and MVP1-4 (Fig. 3), both strains remained present at detectable levels even though population sizes of MVP1-4 declined significantly to approximately log 6 CFU ml^{-1}. Ultimately, it will be necessary to test representative strains of all of the genotypes in combination to identify strains that are especially sensitive to inhibition by others.

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Table 1. Bacterial strains and isolates of 2,4-DAPG-producing fluorescent *Pseudomonas* spp. and *phlD* gene sequences used in this study

Strain	Source[a] (Crop, country)	Genotype[b]	Reference[c]	*phlD* sequence	
				Accession number	Reference[d]
Pf-5	Cotton, USA	A	65	**AF214457**	4
CHA0	Tobacco, Sw	A	47	**AJ278806**	34
MVP1-3	Pea, USA	A	31	**AY928628**	This study
PGNR1	Tobacco, Gh	A	2	**AY928629**	This study
PINR2	Tobacco, It	A	2	**AY928630**	This study
UP61	Birdsfoot trefoil, Ur	A	66	**AF214108**	1
Q2-87	Wheat, USA	B	67	**PFU41818**	17
Q2-87Km[e]	Wheat, USA	B	55	NA[f]	-[d]
Q1-87	Wheat, USA	B	2	**AY928631**	This study
Q4-87, Q9-87, Q12-87, Q13-87	Wheat, USA	B	2	NA	-
Q2-1	Wheat, USA	B	4	**AY928632**	This study
STAD384-97	Wheat, USA	C	31	**AY928633**	This study
STAD376-97	Wheat, USA	C	30	**AY928634**	This study
P2112	Red pepper, Ko	C	3	**AY928635**	This study
Q8r1-96	Wheat, USA	D	21	**AF207693**	4
Q8r1-96Gm[e]	Wheat, USA	D	55	NA	-
L5.1-96	Wheat, USA	D	39	**AY928636**	This study
MVP1-6	Pea, USA	D	31	**AY928637**	This study
FTAD1R34	Wheat, USA	D	30	**AY928638**	This study
ATCC49054	Potato, Russia	D	68	**AY928639**	This study
FFL1R9, Q128-87, Q2-5, Q8r10-96, Q8V8, QT1-5, OC4-1	Wheat, USA	D	30	NA	-
11-18	Maize, France	D[g]	60	**AF396848**	60
4MA30, MVP4-54, MVW1-2, MVW2-2, 2RP17, 1WSU3, 5WSU2	Wheat, Pea, USA	D	31	NA	-
Q2-2	Wheat, USA	E	4	**AY928640**	This study
Q37-87	Wheat, USA	E	2	**AY928641**	This study
QT1-6	Wheat, USA	E	2	NA	-
5MR2	Pea, USA	E	31	**AY928642**	This study
JMP6	Wheat, Net	F	4	**AY928643**	This study
JMP7	Wheat, Net	F	4	**AY928644**	This study

FFL1R18	Wheat, USA	G	4	**AY928645**	This study
CV1-1	Wheat, USA	H	4	**AY928646**	This study
FTAD1R36	Wheat, USA	I	4	**AY928647**	This study
FFL1R22	Wheat, USA	J	4	**AY928648**	This study
FFL1R8	Wheat, USA	J	4	**AY928649**	This study
F113	Sugar beet, Ir	K	16	**AJ278811**	34
1M1-96	Wheat, USA	L	14	**AF207692**	4
W4-4	Wheat, USA	L	4	**AY928650**	This study
1MA1	Pea, USA	L	31	**AY928651**	This study
Phl1C2	Tomato, France	M[g]	U[c]	**AY928652**	This study
D27B1	Wheat, Net	M	4	**AY928653**	This study
PILH1	Tomato, It	M	2	**AJ278810**	34
HT5-1	Wheat, USA	N	4	**AY928654**	This study
7MA12	Pea, USA	O	31	**AY548378**	This study
7MA20	Pea, USA	O	31	**AY928660**	This study
7MA15	Pea, USA	P	31	**AY928655**	This study
6WSU4	Pea, USA	P	31	**AY928656**	This study
4MA6	Pea, USA	P	31	**AY928657**	This study
6MA17	Pea, USA	P	31	**AY928658**	This study
MVP1-4	Pea, USA	P	31	**AY548379**	This study
MVP1-4Km[e]	Pea, USA	P	55	NA	-
MVW1-1	Wheat, USA	P	31	**AY548380**	This study
7MA16, 8MA21, MVP1-5, MVW1-3, 8WSU5	Pea, wheat, USA	P	31	NA	-
MVW4-2	Wheat, USA	Q	31	**AY548381**	This study
MVW4-3	Wheat, USA	Q	31	**AY928659**	This study
FFL2R4	Wheat, USA	T	32	**AY928626**	32
FFL2R5	Wheat, USA	T	32	**AY928622**	32
FFL2R54	Wheat, USA	T	32	**AY928624**	32
FFL2R55	Wheat, USA	T	32	**AY928623**	32
FFL2R61	Wheat, USA	T	32	**AY928625**	32
FFL3R49	Wheat, USA	T	32	**AY928627**	32
42-36	Maize, France	ND[h]	-[c]	**AF396857**	60
42-27	Maize, France	ND	-	**AF396856**	60
39-8	Maize, France	ND	-	**AF396855**	60
37-27	Maize, France	D[g]	-	**AF396854**	60
22-27	Maize, France	ND	-	**AF396853**	60
19-41	Maize, France	ND	-	**AF396852**	60
19-30	Maize, France	ND	-	**AF396851**	60
19-7	Maize, France	ND	-	**AF396850**	60
18-33	Maize, France	ND	-	**AF396849**	60
7-37	Maize, France	ND	-	**AF396847**	60
6-28	Maize, France	ND	-	**AF396846**	60
3-1	Maize, France	ND	-	**AF396845**	60
Q65c-80	Wheat, USA	ND	-	**AJ278807**	34

PITR2	Wheat, It	ND	-	**AJ278809**	34
CM1'A2	Cucumber, Sw	ND	-	**AJ278808**	34
HP72	Bentgrass, Ja	ND	-	**AB125213**	-
HR3-A13	Wheat, USA	PfY	-	**AY391780**	36
PR3-A52	Wheat, USA	PfZ	-	**AY391779**	36
2-79[i]	Wheat, USA	-	69	-	-

[a] Source of fluorescent *Pseudomonas* spp. isolated from the rhizosphere of various crops. Country codes: Gh = Ghana, Ir = Ireland, It = Italy, Ja = Japan, Ko = Korea, Net = Netherlands, Sw = Switzerland, Ur = Uruguay.

[b] Genotype as determined previously by rep-PCR [4, 31] and *phlD*-RFLP analysis [4, 30-32].

[c] References are shown only for strains used for specificity screening of the allele-specific primers. (U) unpublished. (-) not-tested.

[d] Reference where *phlD* sequences were first published. (-) unpublished *phlD* sequence.

[e] Antibiotic-resistant derivatives marked with mini-Tn*7*gfp1 or mini-Tn*7*gfp2 that confer resistance to kanamycin (Q2-87Km, MVP1-4Km) or gentamicin (Q8r1-96Gm), respectively.

[f] NA: non-available *phlD* sequence

[g] Genotype determined by *phlD*-RFLP and rep-PCR with BOXA1R primer (B. B. Landa and D. M. Weller, unpublished results), and confirmed in this study by allele-specific PCR.

[h] ND: Classification by rep-PCR [4, 31] and *phlD*-RFLP analysis [30, 31, 33] was not determined.

[i] *P. fluorescens* 2-79 does not contain *phlD* and was used as a negative control in PCR reactions.

Table 2. PCR primers used in this study

Primer	Sequence	T_m (°C)[a]	Position[b]	Reference
A-align	TGT TGC CGG CTT CCG ACA GGC TGC	86.6	1944-1967	This study
A-gen-exten	GAC ATT CGC ATG GTG ATC GTC ACT	76.0	2545-2571c[c]	This study
B-genlong	CTT GAA TCC CGG CGC CTT ATC G	77.0	2239-2260	This study
D-gen-2	GCA GGA AGT AAG ACC CGG TA	64.2	2212-2231	This study
K-gen	ACC ACC GAC GAC ATT CGA	64.0	2563-2580c	This study
L1-exten	CTC GCT ATC AGG CAG GAA GTA AGA A	79.5	2200-2224	This study
P-gen	CGA GCC AAT GAT TTT GCT	64.5	2403-2421c	This study
B2BF	ACC CAC CGC AGC ATC GTT TAT GAG C	65.6	2640-2664c	33
BPR4	CCG CCG GTA TGG AAG ATG AAA AAG TC	63.4	2036-2061	33

[a] Melting temperature based on the "nearest neighbor" thermodynamic method as predicted by the OLIGO V.6.65 primer analysis software.

[b] Position refers to the sequence of *phl* operon from *P. fluorescens* Q2-87 (accession number PFU41818).

[c] Primer corresponds to complementary DNA strand.

Table 3. PCR conditions for the detection of different genotypes of 2,4-DAPG-producing *Pseudomonas* spp. using allele-specific primers

Target rep-PCR genotype	Allele-specific primers		ΔT_m (°C) [a]	T_{ann} (°C) [b]	Optimal reaction conditions	Amplicon size (bp)
	Upper	Lower				
A	A-align	A-gen-exten	10.6	62.2	1.5 mM $MgCl_2$	628
B	B-genlong	B2BF	2.5	60.0	1.5 mM $MgCl_2$	421
D	D-gen-2	B2BF	1.4	66.0	1.5 mM $MgCl_2$	428
K	BPR4	K-gen	13.5	63.0	1.5 mM $MgCl_2$ + 4% DMSO	544
L	L1-exten	B2BF	0.0	66.0	1.0 mM $MgCl_2$	460
P	BPR4	P-gen	13.0	65.0	1.5 mM $MgCl_2$	385

[a] Difference between melting temperatures (T_m) within a given primer pair based on the "nearest neighbor" thermodynamic method as predicted by the OLIGO primer analysis software.

[b] Optimal annealing temperature found experimentally.

Table 4. Sensitivity of detection of bacterial mixtures growing in broth using allele-specific PCR primers

Treatment [a]		Genotype detected by specific PCR [b]			
Genotypes	Mixture ratio	t=0 h	t=24 h	t=48 h	t=72 h
A	1:0	A	A	A	A
B	1:0	B	B	B	B
D	1:0	D	D	D	D
K	1:0	K	K	K	K
L	1:0	L	L	L	L
P	1:0	P	P	P	P
D + A	1:1	D, A	D, A	D, A	D, A
	1:10	D, A	D, A	D, A	D, A
	10:1	D, ND	D, A	D, A	D, A
	1:100	ND, A	D, A	D, A	D, A
	100:1	D, ND	D, A	D, A	D, A
D + B	1:1	D, B	D, B	D, B	D, B
	1:10	D, B	D, B	D, B	D, B
	10:1	D, B	D, ND	D, ND	D, ND
	1:100	ND, B	D, B	D, B	D, B
	100:1	D, ND	D, ND	D, ND	D, ND
D + K	1:1	D, K	D, K	D, K	D, K
	1:10	D, K	D, K	D, K	D, K
	10:1	D, K	D, K	D, K	D, K
	1:100	ND, K	D, K	D, K	D, K
	100:1	D, ND	D, ND	D, ND	D, K
D + L	1:1	D, L	D, L	D, L	D, L
	1:10	D, L	D, L	D, L	D, L
	10:1	D, ND	D, ND	D, ND	D, ND
	1:100	ND, L	D, L	D, L	D, L

	100:1	D, ND	D, ND	D, ND	D, ND
D + P	1:1	D, P	D, P	D, P	D, P
	1:10	D, P	D, P	D, P	D, P
	10:1	D, P	D, P	D, P	D, P
	1:100	ND, P	D, P	D, P	D, P
	100:1	D, ND	D, P	D, P	D, P
K + P	1:1	K, P	K, P	K, P	K, P
	1:10	K, P	K, P	K, P	K, P
	10:1	K, P	K, P	K, P	K, P
	1:100	ND, P	K, P	K, P	K, P
	100:1	K, ND	K, P	K, P	K, P

[a] Two strains of different genotype were mixed at different ratios in 1/3x KMB broth and grown for 72 h. Samples were taken every 24 h and analyzed by PCR using allele-specific primers for each genotype. Letters indicate the genotype of *Pseudomonas* spp. strains used in each treatment: A, Pf-5; B, Q2-87; D, Q8r1-96; K, F113; L, 1M1-96; P, MVP1-4.

[b] Specific PCR conditions for detecting each genotype are described in Table 3. Letters indicate genotype detected by PCR. ND = below detection limit set for mixed cultures (log 5.7 CFU ml^{-1} for B, D, K and P genotypes, and log 6.7 CFU ml^{-1} for A and L genotypes). The experiment was repeated twice with similar results.

Table 5. Population dynamics of *P. fluorescens* strains F113 and Q8r1-96 on roots of wheat and bulk soil with or without wheat cropping[a]

Sample type	Bacterial treatment	Mean colonization Log(CFU g^{-1} of sample)[b]		AUCPC[c]	
		Q8r1-96	F113	Q8r1-96	F113
Roots and rhizosphere soil (RRS)					
	Q8r1-96	7.91	-----	38.06 a[d]	-----
	F113	-----	7.75	-----	37.42 a
	Q8r1-96 + F113	6.40	7.93	31.32 b	38.24 a
Non-rhizosphere soil (NRS)					
	Q8r1-96	5.78	-----	28.49 a	-----
	F113	-----	6.17	-----	30.42 a
	Q8r1-96 + F113	4.71	5.99	22.99 b	29.37 a
Bulk soil (BS)					
	Q8r1-96	4.77	-----	23.76 b	-----
	F113	-----	5.20	-----	25.77 a
	Q8r1-96 + F113	3.56	5.00	18.02 c	24.88 ab

[a] The experiment was conducted in a growth chamber using non-sterile soil. For details see materials and methods.

[b] Mean colonization was calculated as the mean population density across all cycles [31]. Population data were converted to log CFU/g fresh weight of root.

[c] The area under the colonization progress curve (AUCPC) was calculated using the trapezoidal integration method [56].

[d] Means in the same column followed by the same letter are not significantly different ($P = 0.05$) according to Fisher's protected LSD.

Figure legends.

Fig. 1. Unrooted neighbor-joining tree based on alignment of partial *phlD* DNA sequences from 70 fluorescent *Pseudomonas* spp. strains. Strains are representative of 18 of the 20 rep-PCR DAPG-producing genotypes (letters in parenthesis) identified to date, and representatives of genotypes PfY and PfZ. No letters after strain names correspond to non-determined genotype classification by rep-PCR or *phlD*-RFLP. Bootstrap values are indicated for each branch as percentages. Branches with bootstrap values below 50% were collapsed.

Fig. 2. Detection of *phlD* in different genotypes of 2,4-DAPG-producing *Pseudomonas fluorescens* by allele-specific PCR. Lanes 1 to 10 correspond to samples of purified DNA screened by PCR: 1, Pf-5 (genotype A); 2, CHA0 (genotype A); 3, Q2-87 (genotype B); 4, Q8r1-96 (genotype D); 5, FTAD1R34 (genotype D); 6, F113 (genotype K); 7, 1M1-96 (genotype L); 8, MVP1-4 (genotype P); 9, MVW1-1 (genotype P); and 10, none. Letters on each gel indicate the target genotype detected by each primer pair. Oligonucleotide primers B2BF and BPR4 were "generic" primers designed to amplify *phlD* from all known genotypes of 2,4-DAPG-producing *Pseudomonas* spp. [33]. Primer sequences and PCR conditions are described in Tables 2 and 3. 100-bp DNA ladder from Invitrogen (lanes M) was included in each gel (selected fragment sizes in bp are shown on the left side).

Fig. 3. Inhibition in vitro between genotypes of 2,4-DAPG-producing *Pseudomonas fluorescens*. Strain Q8r1-96Gm (genotype D) was inoculated into 1/3x KMB broth with either Q2-87Km (genotype B) or MVP1-4Km (genotype P) at ratios of 1:1, 1:10, and

10:1. Cultures were incubated at room temperature for 72 h and the density of each strain determined every 24 h by dilution plating. Closed symbols correspond to single inoculation treatments (●, D; ▼, B or P) while open symbols correspond to mixed treatment (○, D; ∇, B or P). The genotype of each strain in a mixture (D:B, D:P) and the relative amounts of each are shown. The experiment was repeated three times. Mean values from a representative experiment are shown.

Fig. 4. Population dynamics of *P. fluorescens* Q8r1-96 and F113 alone and co-inoculated (Q8r1-96+F113) in the wheat rhizosphere (RRS) and in soil where wheat was planted (NRS) or no plants were grown (BS). Natural soil was inoculated with each strain alone or a 1:1 mixture of both strains and seeded (RRS, NRS) or not (BS) to wheat. Bacterial populations in the rhizosphere were measured during 5 consecutive cycles of 3 weeks each. In each graph rhizosphere or soil populations of Q8r1-96 (●,○) and F113 (◇,◆) strain are compared in single (●,◆) and mixed inoculation treatments (○,◇). Population of each strain was determined by PCR using allele-specific primers. Mean values and standard deviations are presented. (*) Indicates populations below detection limit (log 3.26 CFU g^{-1} of sample). Values from mean colonization and AUCPC are shown in Table 5.

Figure 1.

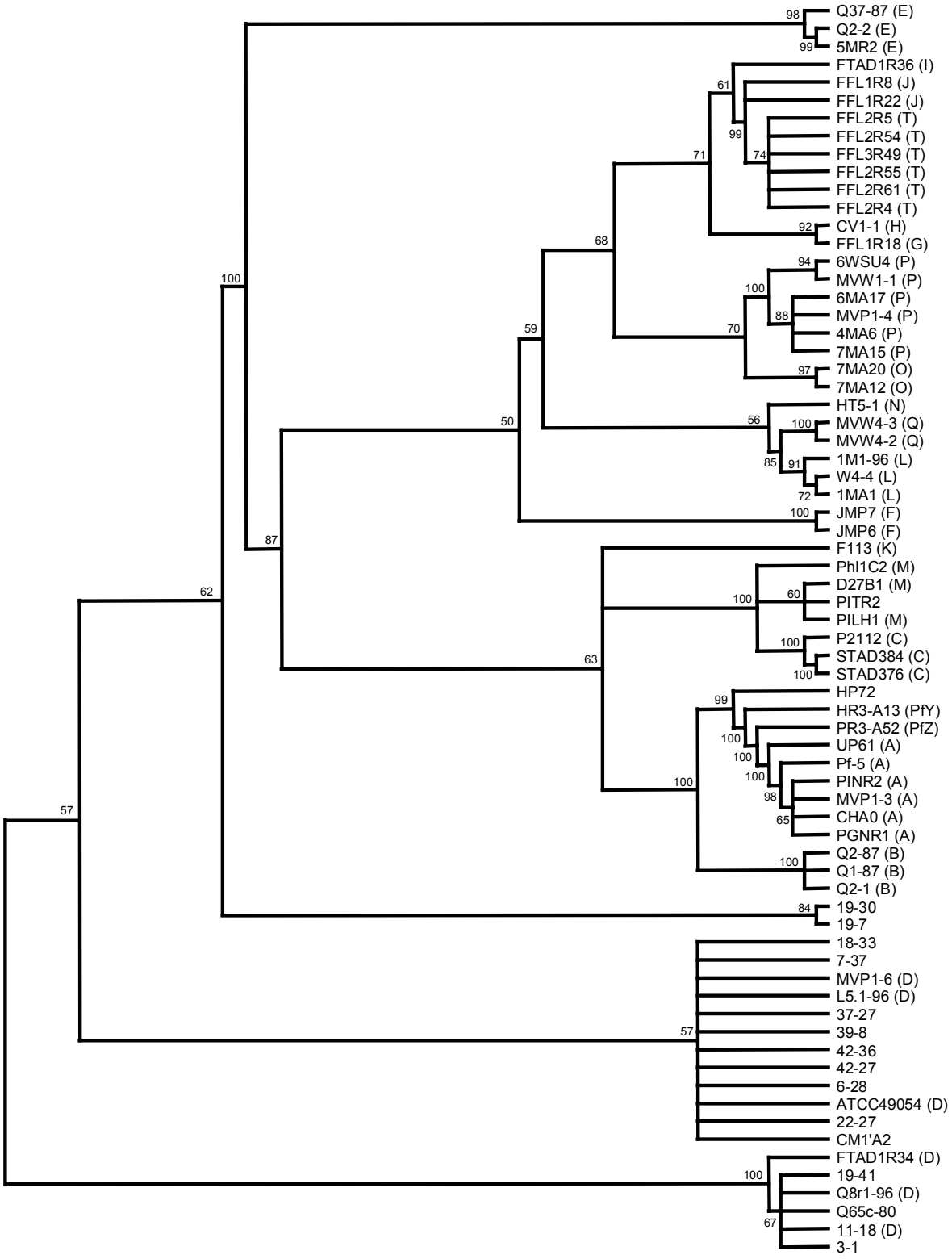

Q37-87 (E)
Q2-2 (E)
5MR2 (E)
FTAD1R36 (I)
FFL1R8 (J)
FFL1R22 (J)
FFL2R5 (T)
FFL2R54 (T)
FFL3R49 (T)
FFL2R55 (T)
FFL2R61 (T)
FFL2R4 (T)
CV1-1 (H)
FFL1R18 (G)
6WSU4 (P)
MVW1-1 (P)
6MA17 (P)
MVP1-4 (P)
4MA6 (P)
7MA15 (P)
7MA20 (O)
7MA12 (O)
HT5-1 (N)
MVW4-3 (Q)
MVW4-2 (Q)
1M1-96 (L)
W4-4 (L)
1MA1 (L)
JMP7 (F)
JMP6 (F)
F113 (K)
PhI1C2 (M)
D27B1 (M)
PITR2
PILH1 (M)
P2112 (C)
STAD384 (C)
STAD376 (C)
HP72
HR3-A13 (PfY)
PR3-A52 (PfZ)
UP61 (A)
Pf-5 (A)
PINR2 (A)
MVP1-3 (A)
CHA0 (A)
PGNR1 (A)
Q2-87 (B)
Q1-87 (B)
Q2-1 (B)
19-30
19-7
18-33
7-37
MVP1-6 (D)
L5.1-96 (D)
37-27
39-8
42-36
42-27
6-28
ATCC49054 (D)
22-27
CM1'A2
FTAD1R34 (D)
19-41
Q8r1-96 (D)
Q65c-80
11-18 (D)
3-1

Figure 2.

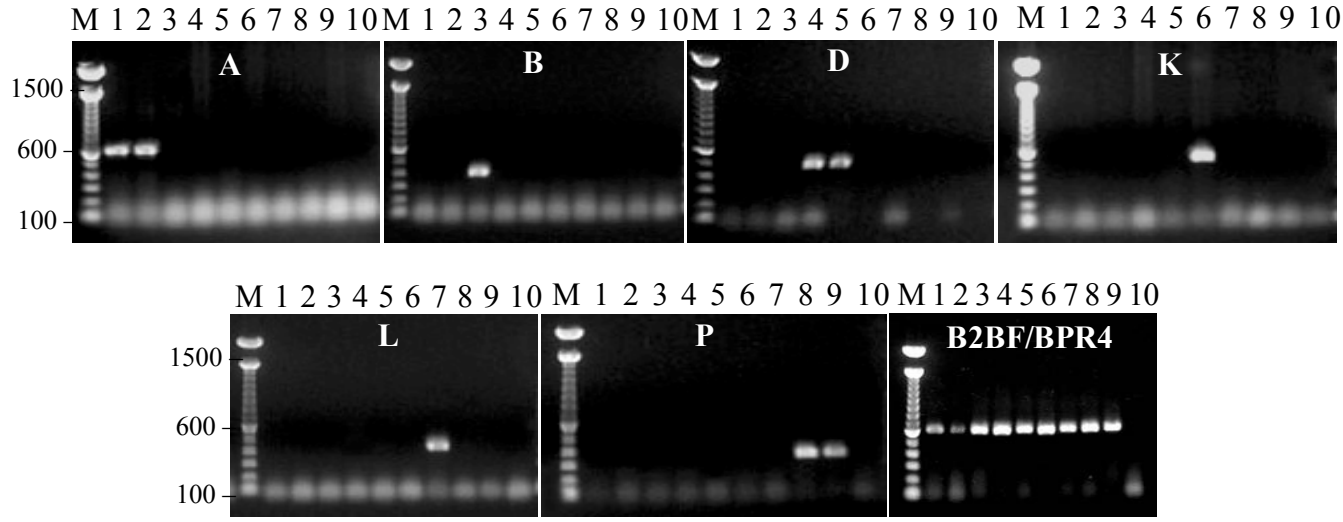


Figure 3.

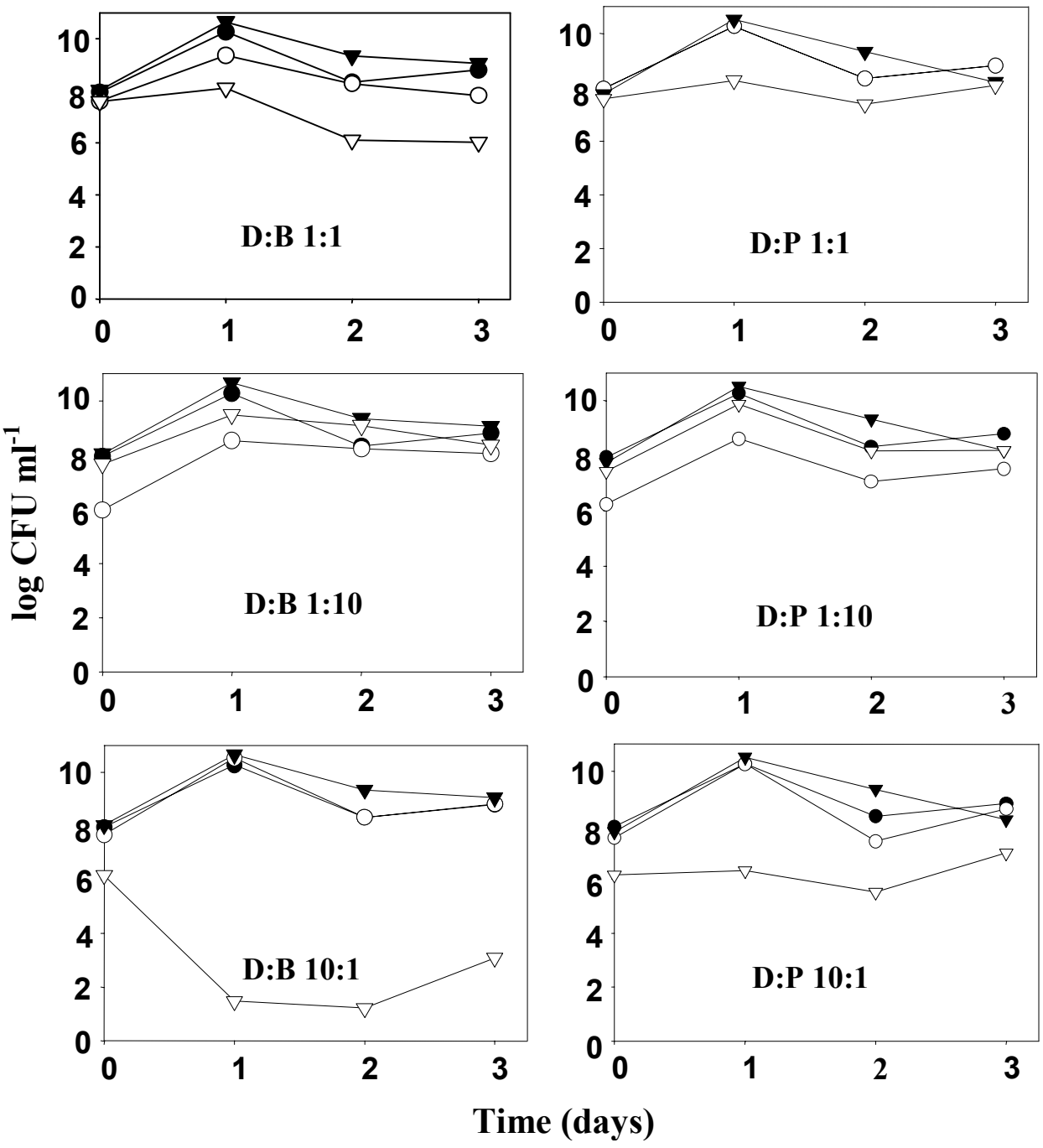
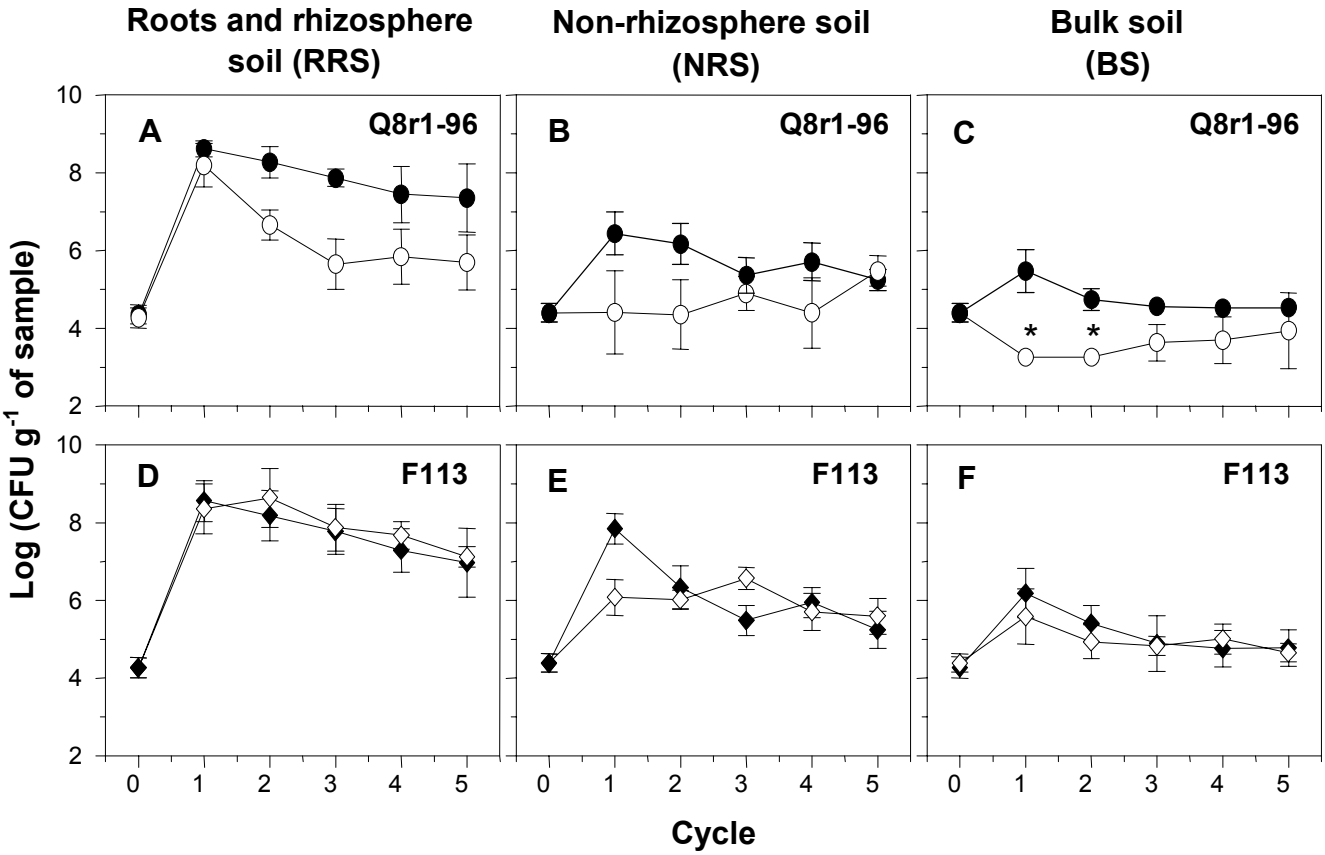


Figure 4.


Roots and rhizosphere soil (RRS)
Non-rhizosphere soil (NRS)
Bulk soil (BS)
A
Q8r1-96
B
Q8r1-96
C
Q8r1-96
D
F113
E
F113
F
F113
Log (CFU g-1 of sample)
Cycle
*
*

CHAPTER 2

Host Crop Affects Rhizosphere Colonization and Competitiveness of 2,4-diacetylphloroglucinol-Producing *Pseudomonas fluorescens*

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Keywords: biological control, fluorescent pseudomonads, microbial ecology, rhizosphere competitiveness, suppressive soils.

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ABSTRACT

Strains of *Pseudomonas fluorescens* producing the antibiotic 2,4-diacetylphloroglucinol (2,4-DAPG) are effective biocontrol agents, and play a key role in the suppressiveness of some soils against soilborne pathogens. In this study we evaluated the effect of the host plant genotype on rhizosphere colonization by indigenous and introduced 2,4-DAPG-producers. Indigenous populations of 2,4-DAPG-producing *Pseudomonas* selected from a Fusarium wilt suppressive soil by the rhizosphere of alfalfa, barley, beans, lentils, lupines, pea, and wheat were above log 5 CFU g^{-1} root, the threshold density previously shown to be required for biocontrol activity. In contrast, flax and oats did not sustain threshold population sizes during six growth cycles in the growth chamber.

A Shano sandy loam was inoculated with all possible combinations of *P. fluorescens* Q8r1-96 (genotype D), F113 (genotype K) and MVP1-4 (genotype P) to study competitive interactions among the strains in the rhizosphere. The three strains were similar in their ability to colonize the rhizosphere of the wheat and pea when inoculated alone into the soil, but when introduced together the outcome of the competition differed according to the host crop. Strain F113 was more competitive than Q8r1-96 on wheat in mixed inoculation, but no significant differences were observed on pea. Strain Q8r1-96 was more competitive than MVP1-4 in mixed inoculation on wheat, but the opposite occurred on pea. In the wheat rhizosphere, F113 reduced the population of MVP1-4 below the detection limit (log 3.26 CFU g^{-1} root), but on pea MVP1-4 was more competitive than F113. When all three strains were present together in the wheat

rhizosphere, F113 outcompeted Q8r1-96 and MVP1-4, but MVP1-4 outcompeted the other two strains in the pea rhizosphere.

Four pea cultivars selected large population sizes (above log 5 CFU g^{-1} root) of indigenous 2,4-DAPG-producing fluorescent *Pseudomonas* from the Fusarium wilt suppressive soil. In addition, eight pea cultivars were grown for five successive cycles in soil inoculated with either MVP1-4 or Q8r1-96. The effect of the pea cultivar on colonization was dependent on the bacterial strain inoculated. MVP1-4 rhizosphere population densities did not differ significantly among pea cultivars, whereas Q8r1-96 populations did. We conclude that the host crop plays a key role in modulating rhizosphere colonization by 2,4-DAPG-producing *Pseudomonas*, especially when strains of different genotypes are present in the same soil, and that this host effect may influence strain survival and biocontrol activity.

INTRODUCTION

Strains of fluorescent *Pseudomonas* spp. have been studied as potential biological control agents since the late 1970s (8). Many of the most effective strains produce one or more diffusible antibiotics active against plant pathogens: for example phenazine derivatives, pyoluteorin, pyrrolnitrin, oomycin A, viscosinamide and 2,4-diacetylphloroglucinol (2,4-DAPG) (23, 56). 2,4-DAPG-producing fluorescent *Pseudomonas* strains isolated worldwide are active against phytopathogenic bacteria, fungi, oomycetes and nematodes (12, 13, 15, 23, 28, 36), and purified 2,4-DAPG has broad antiviral, antibacterial, antifungal, antihelminthic and phytotoxic properties (5, 27, 28). The mechanism of action of 2,4-DAPG against phytopathogens is not fully elucidated, but it was reported to damage membranes and zoospores of *Pythium* spp. (17). The antibiotic can also protect plants indirectly by inducing resistance for example in *Arabidopsis thaliana* against *Peronospora parasitica* (26) and *Pseudomonas syringae* pv. *tomato* (68).

The biosynthetic locus for 2,4-DAPG consists of six genes, *phlF* and *phlACBDE* (5). *phlD*, a key gene for the biosynthesis of 2,4-DAPG, has been the subject of considerable study and encodes a polyketide synthase with similarity to plant genes encoding chalcone/stilbene synthases (5). PhlD plays a key role in the two proposed routes for the biosynthesis of 2,4-DAPG: synthesis of phloroglucinol from glucose (1), or synthesis of the precursor monoacetylphloroglucinol (5). Fluorescent pseudomonads harboring *phlD* (*phlD*$^+$) have been isolated from soils worldwide (16, 29), and DNA sequence analysis shows that this gene is highly conserved among the isolates (16, 29, 44, 57).

Despite extensive research, biocontrol agents account for only 1% of the agricultural chemical sales for plant health management in the USA (20), and only a handful of the biopesticides registered are based on fluorescent *Pseudomonas* strains (20, http://www.epa.gov). One of the main impediments to commercial use of microbial biocontrol agents in the field is inconsistent performance. Many different biotic and abiotic factors such as host plant, soil type and agricultural management regime can affect biocontrol activity (21). These same factors also modulate colonization by introduced rhizobacteria and the microbial community in the soil, which is the main source of competitors for the introduced bacterial inoculants.

Many studies have documented the effect of plant species on microbial communities (3, 4, 40, 48, 69) and on specific groups of bacteria (6, 35, 37, 43). Specific recognition between the plant and symbiotic bacteria such as rhizobia is well known (9, 14, 63) but evidence is growing that specialized relationships also exist between certain non-symbiotic biocontrol bacteria and the host plant (3, 50, 60, 61, 62). Therefore is not surprising that plant species and cultivars differ in their response to introduced and indigenous biocontrol agents, as is evident in evaluations of rhizosphere colonization (32, 33, 44, 51) and biological control activity (41). The specificity of the relationship between 2,4-DAPG-producing fluorescent *Pseudomonas* and the host plant has been suggested at the species (6, 32, 33, 41) and cultivar level (43, 44, 51). Tomato recombinant inbred lines were used to show that three quantitative trait loci (QTL) were associated with disease suppression induced by *Bacillus cereus* UW85. One of these QTL was correlated with population growth of the bacterial inoculant on seeds (62), implying that the host plant facilitates disease suppression by enhancing rhizobacterial growth. Smith and Goodman (62) suggested that identifying host genotypes supporting larger

populations of biocontrol agents would help to improve disease suppression. Other studies have documented the positive relationship between root colonization and disease suppression by *Pseudomonas* (7) and the need for rhizobacteria to reach threshold densities in order to suppress disease (53, 54). Given that the lack of persistence of rhizobacteria in soil and the rhizosphere can be a major impediment to greater use of rhizobacteria in the field (66), considerable research is being conducted to understand the process and molecular basis of rhizosphere colonization (38).

Natural populations of 2,4-DAPG producers are responsible in certain fields for the phenomenon known as take-all decline (TAD) (18, 53, 67): the spontaneous reduction in take-all disease (caused by *Gaeumannomyces graminis* var. *tritici*) severity after several years of barley or wheat monoculture (24, 67). Among the 22 BOX-PCR genotypes of *phlD*$^+$ fluorescent pseudomonads described to date by molecular fingerprinting techniques (named A through T, Pf-Y and Pf-Z) (32, 33, 34, 42, 44, 45), genotype D is the most abundant in TAD soils in Washington State (USA), representing over 50% of the total *phlD*$^+$ population in the wheat rhizosphere (55). *P. fluorescens* Q8r1-96, a strain representative of this genotype, shows exceptional abilities to colonize the rhizosphere of wheat (33, 55) and pea (32). Studies of TAD soils in the Netherlands have shown that genotypes M and F are abundant in those fields (18), but they appear to be absent in Washington TAD soils. Multiple genotypes of 2,4-DAPG producers are found simultaneously in the field (32, 47), but usually one or two are the most abundant, depending on the geographical location (18, 55) and the host crop (34, 47). Nevertheless, little is known about which factors determine the competitiveness among the genotypes of 2,4-DAPG-producing fluorescent *Pseudomonas* present in a soil.

The objectives of this study were to evaluate the effect of host plant genotype on i) rhizosphere colonization by indigenous and introduced 2,4-DAPG producing fluorescent *Pseudomonas*, and ii) competition among strains of different BOX-PCR genotypes. We show that alfalfa, beans, barley, lentils, lupines, pea and wheat selected higher indigenous populations of 2,4-DAPG producers from a Puget silt loam than did oats and flax. Furthermore, pea cultivars were found to differ in the capacity to select for indigenous 2,4-DAPG producers from the soil, and an effect of pea cultivar on the rhizosphere colonization by different introduced strains also was observed. Finally, competition among 2,4-DAPG-producing *P. fluorescens* strains on wheat and pea was greatly affected by the host crop.

MATERIALS AND METHODS

Bacterial strains and growth media. *Pseudomonas fluorescens* strains used in this study produce the antibiotic 2,4-DAPG: Pf-5 (genotype A, 25), Q2-87 (genotype B, 65), Q8r1-96 (genotype D, 54), MVP1-6 (genotype D, 32), F113 (genotype K, 58), 1M1-96 (genotype L, 55), MVP1-4 (genotype P, 32), and MVW1-1 (genotype P, 32). Spontaneous rifampicin-resistant mutants of *P. fluorescens* strains Q8r1-96, F113 and MVP1-4 were used in growth chamber studies (32, 33).

All strains were cultured at 28^{o}C in one-third-strength King's medium B (1/3x KMB) broth or agar (31). Stock cultures were stored in 1/3x KMB broth plus 20% glycerol at -80ºC. When appropriate, antibiotics were added to media at the following concentrations: ampicillin (Amp), 40 μg ml^{-1}; chloramphenicol (Cm), 13 μg ml^{-1}; cycloheximide (Chex), 100 μg ml^{-1}; and rifampicin (Rif), 100 μg ml^{-1}. One-tenth-strength

tryptic soy broth (TSB, Difco®) supplemented with Chex (1/10x TSB+) was used for quantification of total culturable heterotrophic bacteria.

Selection of indigenous 2,4-DAPG-producers by crop species. A Puget silt loam was collected from the upper 30 cm of the soil profile from a field located at the Washington State University Research and Extension Center (Mount Vernon, Washington), air dried, sieved and stored at room temperature in plastic buckets. The field has been cropped continuously to pea for over 30 years, is suppressive to *Fusarium* wilt of pea caused by *Fusarium oxysporum* f. sp. *pisi* (32), and contains 2,4-DAPG-producing fluorescent *Pseudomonas* genotypes A, D, L, O, P and Q, which were detected when the soil was planted to pea or wheat (32). Two hundred grams of soil were added to pots (7x7x6.5 cm) and planted to one of nine crops. For all legumes, 6 seeds were sown per pot and for the other crops, 10 seeds were sown per pot: alfalfa (*Medicago sativa* L. cv. Magnum IV), barley (*Hordeum vulgare* L. cv. Baronesse), bean (*Phaseolus vulgaris* L. cv. Red Hoak), flax (*Linum usitatissimum* L. cv. Norlin), lentil (*Lens culinaris* Medik. cv. Brewer), lupine (*Lupinus albus* L. cv. 98LI8535), oat (*Avena sativa* L. cv. Caliber), pea (*Pisum sativum* L. cv. Columbian), and wheat (*Triticum aestivum* L. cv. Penawawa). Plants were incubated in a growth chamber at 22 ± 1°C, with a 12-h photoperiod and 40 to 60% relative humidity. Soil in each pot was cropped to six successive cycles of the same plant species, with each cycle lasting 3 weeks, as described by Landa et al. (33). Each treatment was replicated three times, with each pot serving as a replicate, and the experiment was arranged in a completely randomized design. Each pot was watered at the beginning of each cycle (see below) with 50 ml of a suspension of metalaxyl (Syngenta, Greensboro, NC) at 2.5 mg/ml active ingredient to control *Pythium* root rot. Pots were watered every two days with tap water, and treatments with non leguminous plants were

watered once a week with 50 ml of a 1x Miracle-Gro fertilizer solution (Scotts, Marysville, OH).

After three weeks (one "cycle"), plants were harvested and rhizosphere population density of indigenous 2,4-DAPG producers was determined (see below) on six randomly selected plants (two from each replicate pot). Shoots of the remaining plants of each crop were excised, and the soil and associated roots were decanted into a plastic bag and mixed by shaking. The soil was returned to the pots and sown again to begin the next cycle. All treatments were maintained for a total of six 3-week cycles and roots were assayed for the presence of introduced bacteria after each cycle.

The population size of 2,4-DAPG producers was determined by using a PCR-based dilution endpoint technique (46). This technique is based on the specific detection of *phlD* by PCR after serially-diluted root washes are incubated in a selective growth medium. Briefly, root washes were serially diluted in sterile deionized water and transferred to 1/3x KMB containing Chex, Cm and Amp (KMB^{+++}). The terminal dilution cultures (TDC) were determined as the greatest dilution showing bacterial growth (absorbance at 600 nm (OD_{600nm}) $\geq$ 0.05). Microtiter plates containing dilutions on KMB^{+++} were frozen at -20°C prior to analysis. Detection of $phlD^{+}$ was conducted by PCR using the "generic" primers B2BF and BPR4 (46) designed for *phlD*-based detection of 2,4-DAPG producers. Samples were analyzed starting from the TDC and continuing into lower dilutions until at least three consecutive *phlD*-specific amplification products were detected in three consecutive wells. When amplification of *phlD* by PCR did not occurr in any of the dilutions, the population density for that sample was set at the detection limit of the technique, log 3.26 CFU g^{-1} root.

Rhizosphere colonization of wheat and pea by 2,4-DAPG-producing *P. fluorescens* strains. A Shano sandy loam from a noncropped site near Quincy, Washington, USA (Quincy virgin soil) was used to test the effect of crop species on the interactions among *P. fluorescens* strains Q8r1-96 (genotype D), F113 (genotype K), and MVP1-4 (genotype P). These strains were selected because of their superior rhizosphere competence on wheat and/or pea (32, 33, 34). A previous study (16) showed that no inhibition occurs between these strains when grown together in broth culture, which allowed us to quantify the rhizosphere populations by a technique involving growth of both strains in the same culture (see below). Bacteria were introduced into soil as a suspension in 1% methylcellulose (33) at 100 ml of suspension per kg of soil. Single inoculation treatments consisted of soil inoculated with *P. fluorescens* strains Q8r1-96, F113 or MVP1-4 (approximately $1x10^4$ CFU g^{-1} soil for each strain). Mixed treatments consisted of pairwise mixtures of Q8r1-96 plus F113, Q8r1-96 plus MVP1-4, and F113 plus MVP1-4 (each component of the mixture at approximately $0.5x10^4$ CFU g^{-1} soil); and a triple mixture of Q8r1-96, F113 and MVP1-4 (each at approximately $0.33x10^4$ CFU g^{-1} soil). A control soil received only a sterile 1% methylcellulose solution. Population densities of each strain established in the soil ("cycle 0") were determined by assaying three 0.5-g samples from each inoculated soil by a modification of the PCR-based dilution endpoint assay (16) described below. Soils were added to pots (7x7x6.5 cm, 200 g/pot) and either wheat (cv. Penawawa) or pea (cv. Columbian) was sown (6 seeds/pot) with seeds pre-germinated on moist paper at room temperature for 1 day. Pots were incubated in growth chambers at 15 ± 1°C (wheat) or 22 ± 1°C (pea), with a 12-h photoperiod and 40 to 60% relative humidity. Each pot was watered at the beginning of each cycle with 50 ml of a suspension of metalaxyl as described above. Wheat was

watered every other day and pea daily with tap water, and both crops were watered once a week with 50 ml of a 1x Miracle-Gro fertilizer solution. Soils were cycled with wheat or pea five times, with each cycle lasting 3 weeks. Each treatment was replicated three times with one pot serving as a replicate, and the experiment was arranged in a completely randomized design. The experiment was repeated three times with similar results.

Total culturable heterotrophic bacteria were quantified in 1/10x TSB^{+} broth as previously described (33). Populations of introduced *P. fluorescens* strains were enumerated in 1/3x KMB^{+++} containing Rif (KMB^{+++}Rif) by using a modification (16) of the PCR-based dilution endpoint assay (46) that employs allele-specific PCR amplification of *phlD* and allows the simultaneous detection of strains of different 2,4-DAPG genotypes in soil samples. All PCR reactions were carried out according to De La Fuente et al. (16). Primers D-gen-2 and B2BF were used for detection of the D-genotype strain Q8r1-96; BPR4 and K-gen for the K-genotype strain F113; and BPR4 and P-gen for the P-genotype strain MVP1-4 (16). The TDC of all treatments were analyzed by allele-specific PCR with all three sets of primers. Additional PCR reactions were performed with samples from lower dilutions until an amplification product was detected in at least three consecutive samples.

Rhizosphere colonization of pea cultivars by 2,4-DAPG-producing indigenous populations and strains Q8r1-96 and MVP1-4. Two cycling experiments were carried out in the greenhouse as described above to assess the effect of pea cultivars on the colonization of indigenous (Experiment I) or introduced (Experiment II) 2,4-DAPG-producing *Pseudomonas*. For Experiment I, four pea cultivars including Columbian, Little Marvel, Quincy and WSU31 were sown (6 seeds per pot) in Puget silt loam from

Mount Vernon referred above. For Experiment II, Quincy virgin soil was inoculated with *P. fluorescens* Q8r1-96, MVP1-4 (approximately $1x10^4$ CFU g^{-1} soil each) or a sterile solution of methylcellulose (non-inoculated control) and added (200 g) to pots. The pea cultivars Alaska, Columbian, Joel, Little Marvel, Majorette, Quincy, Rex and WSU31 were sown (6 seeds per pot) and pots were incubated in a growth chamber at 22 ± 1°C, with a 12-h photoperiod and 40 to 60% relative humidity. Each pot was watered at the beginning of each cycle with 50 ml of a suspension of metalaxyl, daily with tap water, and once a week with 50 ml of a 1x Miracle-Gro fertilizer solution. Each treatment was replicated three times, with one pot serving as a replicate, and the experiment was organized in a completely randomized design. Peas were grown for a total of two (Experiment I) or four (Experiment II) 3-week cycles and the roots were assayed for bacteria after each cycle as described above. Population sizes of indigenous (Experiment I) and introduced (Experiment II) bacteria were determined by the PCR-based dilution endpoint assay, using 1/3x KMB^{+++} (Experiment I) or 1/3x KMB^{+++}Rif (Experiment II) as a selective medium. For the detection of total $phlD^+$ populations PCR reactions were carried out with "generic" primers B2BF and BPR4. For experiment II, RFLP analysis of the *phlD* amplified fragment was performed using the enzyme *Hae*III (42) to discriminate among BOX-PCR genotypes of introduced strains. When contamination with a non-inoculated genotype was detected (Experiment II), the allele specific PCR technique (16) was used to detect the subordinate strain as described above.

Growth kinetics in vitro at different incubation temperatures. Because the rhizosphere competitiveness assays on wheat and pea were performed in growth chambers set at different temperatures, we studied the effect of the incubation temperature (15°C and 22°C) on the growth kinetics of *P. fluorescens* strains Pf-5, Q2-

87, Q8r1-96, MVP1-6, F113, 1M1-96, MVP1-4 and MVW1-1, which are representatives of the most studied genotypes of 2,4-DAPG producers. Overnight cultures of each strain in 1/3x KMB broth were harvested by centrifugation in a microfuge (10,000 x g, 5 min), suspended in the same media, and the concentration of the bacteria was adjusted to an OD_{600nm} of 0.1 by using a SAFIRE microplate reader (Tecan Systems, Inc., San Jose, CA). Suspensions were diluted 1:10 with 1/3x KMB broth (approximate starting concentration $1x10^7$ CFU ml^{-1}) and added (200 µl per well) to 96-well microtiter plates. Samples were replicated three times and the entire experiment was conducted twice. The plates were incubated in incubators set at 15 and 22°C for 47 h and bacterial growth was monitored at 1, 2 or 6-h intervals with the SAFIRE microplate reader. Data from OD_{600nm} values were converted to log CFU ml^{-1} by using the relationship 1 OD_{600nm} = $1.53x10^9$ (L. De La Fuente, unpublished)

Data analysis. Population density data from greenhouse studies were converted to log CFU per gram of root fresh weight to satisfy the assumptions of analysis of variance (ANOVA). For graphical representation of the data the log of the arithmetic means was used. Differences in population densities among treatments were determined by standard ANOVA and mean comparisons among treatments were performed by Fischer's protected least significance difference (LSD) test at $P = 0.05$ by using the STATISTIX 8.0 software (Analytical Software, St. Paul, MN). Initial ANOVA analysis was performed as a factorial design (bacterial treatment, host crop and cycle). Due to significant ($P < 0.05$) interactions among these three factors, ANOVA was performed independently for each host crop, cycle and specific bacterial treatments. Kruskal-Wallis analysis was performed whenever ANOVA assumptions were violated. Area under the colonization progress curve (AUCPC), previously used as a general measure of

rhizosphere competence (32), was calculated by using the trapezoidal integration method (10). Mean colonization was calculated as the mean population density across all cycles (32). For rhizosphere samples in which no PCR signal was detected, population densities were set at the detection limit of the assay used (log 3.26 CFU g^{-1} of root for both the PCR-based dilution endpoint (46) and the allele-specific PCR technique (16).

RESULTS

Selection of indigenous populations of 2,4-DAPG-producing fluorescent *Pseudomonas* by different crops. 2,4-DAPG-producing fluorescent *Pseudomonas* were detected in the rhizospheres of all crops (alfalfa, barley, bean, flax, lentil, lupine, oat, pea and wheat) grown in Puget silt loam, but population sizes differed significantly among crops (Fig. 1, Table 1). In general, on the basis of AUCPC, mean colonization values and population densities at each cycle, legumes (alfalfa, beans, lentils, lupines, and peas) supported the largest populations of 2,4-DAPG producers, followed by barley and wheat; flax and oats were significantly less supportive than the other crops (Fig. 1, Table 1). AUCPC for lentils was significantly ($P < 0.05$) greater than that for all of the other crops, while AUCPC values for flax and oats were significantly lower (Table 1). All crops except oats and flax supported population densities of 2,4-DAPG producers above the threshold density of log 5 CFU g^{-1} root known to be required for take-all suppression in wheat (54). However, crops differed significantly in the rate at which threshold densities developed in the rhizosphere. At the end of the first cycle of growth, densities of 2,4-DAPG producers on alfalfa, barley, flax, oats and wheat were either at or below log 5 CFU g^{-1} root, whereas densities on bean, lentil, lupine and pea were much greater: log

7.4, 5.8, 5.9, and 6.4 CFU g^{-1} root, respectively. By the end of cycle 2, only population densities on barley, flax and oat were below log 5.0 CFU g^{-1} root (Fig. 1). Oats and flax did not support population densities above the threshold in four of the six cycles of the experiment (Fig. 1). The crops also varied in the frequency of root samples for which 2,4-DAPG producers were above the detection limit of log 3.26 CFU g^{-1} root, mostly during cycles 1 and 2. The percentage of individual root samples (n=36 for each crop) for which a positive detection of *phlD* was obtained over the six cycles for alfalfa, barley, bean, flax, lentil, lupine, oat, pea and wheat was 83, 92, 94, 64, 92, 86, 61, 97 and 86%, respectively.

Growth of 2,4-DAPG producers at 15°C and 22°C. Growth kinetics in vitro were determined in order to evaluate a possible effect of the incubation temperature on the relative growth of *P. fluorescens* strains Q8r1-96, F113 and MVP1-4. Relative growth of eight *P. fluorescens* strains including Q8r1-96, F113 and MVP1-4 did not differ at incubation temperatures of 15°C (Fig. 2, A), or 22°C (Fig. 2, B). All strains grew faster at 22°C. At both temperatures, the slowest growing strain was Q2-87, and the fastest was Pf-5. Similar results were obtained when the experiment was repeated. These results allowed us to use different optimal incubation temperatures for wheat and pea in our competition assays.

Competitiveness of *P. fluorescens* Q8r1-96, F113 and MVP1-4 in the rhizosphere of wheat and pea. Wheat and pea were used to assess whether the host crop modulates interactions among 2,4-DAPG-producing strains in the rhizosphere. Competition among *P. fluorescens* Q8r1-96, F113 and MVP1-4 was affected by the host crop. When strains

Q8r1-96 and F113 were introduced separately into soil that was then planted to wheat or pea, no significant differences in population densities occurred from each other over the five cycles. In the pea rhizosphere, the density of Q8r1-96 was significantly greater than that of F113 only in cycle 3 (Fig. 3, solid bars graphs A and B). However, AUCPC values (Table 2), showed that F113 colonized wheat better than did Q8r1-96, and that both strains colonized pea equally well. When strains Q8r1-96 and F113 (Fig. 3, dashed bars graphs A and B) were present together in the wheat rhizosphere, the population size of F113 was significantly greater than that of Q8r1-96 in the third, fourth and fifth cycles, indicating that F113 was more competitive. However, for mixed inoculations in the pea rhizosphere, densities of the two strains did not differ significantly in any cycle, indicating that the strains are equally competitive on pea. AUCPC values confirmed these results. The AUCPC value for F113 in mixed inoculations (Q8r1-96+F113) on wheat was significantly ($P < 0.05$) higher than that for Q8r1-96, but the values for the two strains on pea did not differ (Table 2).

When strains Q8r1-96 and MVP1-4 were introduced separately into soil, no differences occurred in the population densities of the two strains attained in the wheat rhizosphere. However, MVP1-4 colonized the pea rhizosphere significantly better than Q8r1-96 during the last two cycles of the experiment (Fig. 3, solid bars graphs C and D). The AUCPC for Q8r1-96 was greater than that of MVP1-4 on wheat and the opposite was observed on pea (Table 2). When strains Q8r1-96 and MVP1-4 were present together into the wheat rhizosphere, the population size of Q8r1-96 was significantly greater than that of MVP1-4 (Fig. 3, dashed bars graphs C and D) in cycles 1, 2, 3 and 4, indicating that Q8r1-96 outcompeted MVP1-4. Differences between the population sizes of the two strains were as great as log 3 CFU g^{-1} root in cycle 3. However, when the two strains

were present together in the pea rhizosphere, the opposite occurred and MVP1-4 outcompeted Q8r1-96; the population size of MVP1-4 was significantly greater than that of Q8r1-96 in cycles 3, 4 and 5 (Fig. 3, dashed bars graphs C and D). The AUCPC value for MVP1-4 in mixed inoculation (Q8r1-96+MVP1-4) was significantly higher ($P < 0.05$) than the value for Q8r1-96 on pea, but the opposite was observed on wheat (Table 2).

When strains F113 and MVP1-4 were introduced separately into soil, only small differences in population densities of the two strains occurred in the wheat and pea rhizospheres. In the wheat rhizosphere, the population size of F113 was significantly greater than that of MVP1-4 during cycles 1 and 2, and in the pea rhizosphere MVP1-4 was significantly greater than F113 at cycle 5 (Fig. 3, solid bars graphs E and F). The AUCPC value for F113 was greater than that of MVP1-4 in the wheat rhizosphere but the opposite occurred in the pea rhizosphere (Table 2). When F113 and MVP1-4 were introduced into soil together, F113 strongly outcompeted MVP1-4 in the wheat rhizosphere (Fig. 3, dashed bars graphs E and F). The population density of MVP1-4 declined steadily, was significantly lower than the density of F113 in all five cycles, and dropped below the detection limit (log 3.26 CFU g^{-1} root) during cycle 5. In contrast, in the pea rhizosphere the population sizes of the two strains were equivalent except in cycles 2 and 5, when the density of MVP1-4 was significantly ($P < 0.05$) greater than that of F113 (Fig. 3, dashed bars graphs E and F). The AUCPC value for F113 in mixed inoculation (F113+MVP1-4) was significantly higher ($P < 0.05$) than that of MVP1-4 on wheat, but the opposite was observed on pea (Table 2).

When strains Q8r1-96, F113 and MVP1-4 were mixed together into soil, F113 strongly outcompeted MVP1-4 and Q8r1-96 (Fig. 3, graphs G and H) in the wheat

rhizosphere. The density of F113 was 1,000-fold greater than that of Q8r1-96 in cycle 5, and the population size of MVP1-4 dropped below the detection limit in cycles 1 and 5. The population densities of the three strains in the pea rhizosphere were substantially different from those in the wheat rhizosphere (Fig. 3, graphs G and H). The population density of MVP1-4 was significantly greater than that of Q8r1-96 in cycles 2 through 5 and greater than that of F113 in cycles 2 and 4. The population density of strains Q8r1-96 and F113 declined from cycle 3 to 5. The AUCPC value for MVP1-4 was the lowest ($P < 0.05$) of the three strains in the wheat rhizosphere, but the value for MVP1-4 was the highest on pea, while values for Q8r1-96 and F113 did not differ significantly ($P > 0.05$) on either crop (Table 2).

Rhizosphere samples from all treatments were inoculated into 1/10x TSB^+ broth to determine the density of the total aerobic culturable heterotrophic bacteria. No differences were detected among all treatments on each crop (Fig. 4). Total bacterial populations also were similar between wheat and pea (Fig. 4, A and B).

Effect of cultivar on pea in rhizosphere colonization. Population densities of indigenous fluorescent *Pseudomonas* and 2,4-DAPG-producing *Pseudomonas* differed among the four pea cultivars (Fig. 5). In both of two cycles, 'Little Marvel' supported the highest ($P < 0.05$) populations of both indigenous and 2,4-DAPG-producing *Pseudomonas*. WSU31 and Columbian cultivars supported intermediate and 'Quincy' the lowest populations of 2,4-DAPG-producing *Pseudomonas* (Fig. 5B).

To further assess the effect of pea cultivar on rhizosphere colonization, strains Q8r1-96 and MVP1-4 were added separately to soil and eight pea cultivars were then cycled. Strain MVP1-4 colonized the rhizosphere of all cultivars in all cycles to the same extent

(Fig. 6B). In contrast, significant differences in the population density of Q8r1-96 in the rhizosphere of the eight pea cultivars were detected (Fig. 6A); however those differences were not consistent across all four cycles. For example, the population densities of Q8r1-96 on 'Little Marvel' and 'Alaska' differed by log 2.3 and 1.6 CFU g^{-1} root in cycles 1 and 2, respectively, but no differences occurred in cycles 3 and 4 (Fig. 6A).

A non-desired contamination by MVP1-4 was observed in the fourth cycle in soils inoculated originally with Q8r1-96 and seeded with the pea cultivars Alaska, Joel, Majorette and WSU31. MVP1-4 was detected in the TDC as the most abundant strain in the rhizosphere of those four cultivars. An analysis using allele-specific PCR showed that the density of Q8r1-96 was approximately log 1 CFU g^{-1} root below the density of MVP1-4.

DISCUSSION

Growth promotion of crop species by beneficial rhizobacteria requires a close interaction between the bacteria and roots of the host. A relationship with a high degree of specificity was observed for *Rhizobium* as early as 1928, when effective nodulation of a pea cultivar was achieved only by inoculation with a particular group of rhizobial strains (22). Nodulation effectiveness involved specific recognition between *nodX* in *Rhizobium leguminosarum* bv. *viciae* and *sym-2* in pea (14), a relationship resembling the gene-for-gene theory described for plant pathogens (19). A variety of studies have described a specific influence of the plant genotype on non-symbiotic beneficial bacteria. Substitution of a single pair of chromosomes in spring wheat modified the composition of the rhizosphere microflora (50) and the number of antagonistic bacteria on their roots (3).

More recently, the biocontrol activity of *B. cereus* against *Pythium torulosum* on tomato was partially explained by three QTL in the host (62).

In this study we focused on the effect of the host on rhizosphere colonization by 2,4-DAPG-producing fluorescent pseudomonads. First we determined the ability of nine crops to select indigenous populations of 2,4-DAPG-producing pseudomonads. In general, 2,4-DAPG producers survive at densities below the detection level in bulk soil, but rapidly proliferate when a crop is grown. Pea monoculture over a span of 30 years in a field at Mount Vernon, Washington, has enriched for a population of 2,4-DAPG producers that rapidly responds to plant roots. Thus, the Puget silt loam was an ideal medium to compare the supportiveness of different crop rhizospheres to these bacteria. The nine crops differed in the size of the populations of 2,4-DAPG producers supported over time and the rate at which threshold densities (log 5 CFU g^{-1} root) were reached. All legumes (alfalfa, beans, lentils, lupines and pea) and most cereals (wheat and barley) supported larger populations of *phlD*$^+$ pseudomonads during the cycling experiments performed in a growth chamber than did oats and flax (Fig. 1, Table 1). Flax and oats did not sustain indigenous *phlD*$^+$ populations above log 5 CFU g^{-1} root (Fig. 1) throughout the experiment. Both of these crops had the lowest AUCPC, mean colonization values (Table 1), and frequency of *phlD*$^+$ detection (about 60%). The lower supportiveness of the oat rhizosphere to 2,4-DAPG producers previously was observed (54), and is demonstrated again in our study (Fig. 1, Table 1). Bergsma-Vlami et al. (6) reported that lily was the least supportive of *phlD*$^+$-strasins of four crops tested. Of interest is the fact that both oats and species of Liliaceae are known for producing steroid saponines. For example, the saponines avenacoside A and B (39), produced by oats in the rhizosphere, have broad spectrum antimicrobial activity and may be a factor in the low supportiveness

of oats to 2,4-DAPG-producers. In a previous study, flax supported populations of indigenous *phlD*$^+$ above log 5 CFU g^{-1} root when grown in soils continuously cultivated to wheat or flax (34). The fact that we obtained different results with flax suggests that the cropping history of the soil may affect the selection of 2,4-DAPG producers by the roots of specific crops. Unfortunately, we were unable to consistently determine the most abundant genotypes present in the rhizosphere of the different crops, probably due to the fact that samples were stored for two years at -20°C prior to analysis. Nevertheless, whenever we succeeded, *phlD*-RFLP profiles corresponding to genotype D generally were largely the most abundant (data not shown).

Recent studies have conclusively demonstrated that the plant species modulates the population size and composition of indigenous or introduced 2,4-DAPG-producing fluorescent *Pseudomonas* genotypes (6, 34, 47, 52). However, no information is available on whether the host plant affects competition among genotypes of 2,4-DAPG producers in the rhizosphere. This is an important question because multiple BOX-PCR genotypes of 2,4-DAPG producers commonly occur in the field, yet only one or two may dominate in the rhizosphere of a certain crop, especially in disease-suppressive soils (18, 32, 34, 47). Using the allele-specific primers, we were able to address the question of competition among genotypes at a very fine scale in the wheat and pea rhizospheres. We showed for the first time that the host plays a key role in the competition among genotypes of 2,4-DAPG producers (Fig. 3, Table 2). The three strains used in this study were selected because they are known to be aggressive "premier" colonists of the wheat and/or pea rhizosphere (32, 33, 34). When inoculated alone, all three strains showed rhizosphere population dynamics in wheat and pea typical of a premier strain, but they were not identical in their colonization of these two crops (Fig. 3). AUCPC values

showed that F113 was slightly more aggressive on wheat than Q8r1-96 and MVP1-4, and MVP1-4 was more aggressive on pea (Table 2). However, when the strains were introduced together the strong influence of the host on competition was evident (Fig. 3). In the wheat rhizosphere, F113 outcompeted Q8r1-96 (Fig. 3), consistent with the findings of Landa et al. (33). However, we were surprised to find that the antagonism of Q8r1-96 by F113 did not occur in the pea rhizosphere. The most striking effect of the plant host on inter-genotype competition occurred with MVP1-4. When applied alone, strain MVP1-4 aggressively colonized wheat, but when placed in competition with F113 and/or Q8r1-96, it became a very poor colonizer and its density even dropped below the detection limit at times (Fig. 3). In striking contrast, in co-inoculation studies on pea, strain MVP1-4 outcompeted strains Q8r1-96 and F113 (Fig. 3, Table 2). The aggressive competitiveness of the P-genotype against other genotypes in the pea rhizosphere is further supported by earlier studies of Landa et al. (32), which showed that strain MVP1-4 displaced Q-genotype strain MVW4-2 when MVP1-4 contaminated soil originally inoculated with MVW4-2. Similarly, in our study, MVP1-4 displaced Q8r1-96 on the pea cultivars Alaska, Joel, Majorette and WSU31 after the fourth cycle, in soils originally inoculated with Q8r1-96 (Fig. 6). These findings are especially interesting because Q8r1-96, F113 and MVP1-4 are strains of *P. fluorescens* and share a very similar genetic background.

Two key unanswered questions are why one BOX-PCR genotype outcompete another on a certain host and the mechanism involved. One possibility is direct antagonism of one strain by another. Validov et al. (64) screened a collection of 2,4-DAPG-producing fluorescent pseudomonads and showed that over 70% of the isolates had antagonistic activity against other isolates in vitro, and in some cases this activity was explained by

the production of bacteriocins (64). However, additional studies showed that Q8r1-96, F113, and MVP1-4 were not antagonistic in vitro against each other upon induction for bacteriocin production (S. Validov and D.M. Mavrodi, unpublished data). Nevertheless, differential expression of antagonistic factors in the pea and wheat rhizosphere cannot be ruled out. A second possibility is that the composition and amount of root exudates (23), or the root topology of a particular host favors the growth of one genotype over another. Although this explanation is appealing, it should be remembered that different genotypes defined by BOX-PCR differ little in their carbon utilization profiles (45, 55). However, classical carbon and nitrogen utilization profiles probably do not reflect the full range of organic substrates in rhizodeposition. Studies conducted to date do not show much difference among genotypes in growth on seed and root exudates of wheat and pea (L. De La Fuente and D.M. Weller, unpublished). A third possibility is that some genotypes have an affinity for the roots of a particular crop. Unknown rhizobacterial or plant traits would allow one genotype to more efficiently colonize the roots of the host plant. Finally, an effect of other rhizosphere microflora on the interaction between the roots and strains of *Pseudomonas fluorescens* cannot be discarded.

The positive relationship between root colonization and biocontrol activity by introduced or indigenous rhizobacteria is now well established (11, 32, 33, 54, 55). The activity of the total rhizosphere microflora is considered a major biotic factor that can affect the colonization and biocontrol activity of rhizobacteria (66). However, our results suggest that the amount of total culturable bacteria played little role in the differential competitiveness of strains Q8r1-96, F113 and MVP1-4 in the pea and wheat rhizosphere because the population size of the total bacterial population did not differ among treatments and crops (Fig. 4). Our results further suggest that in biocontrol studies, the

failure of introduced rhizobacteria to establish and persist in the rhizosphere may result more from very specific competition from indigenous, closely related strains with the same biocontrol trait and overlapping niche, than from general competition with the total microflora. This scenario could explain the variability in colonization and disease suppression that often occurs when rhizobacteria are applied to soils which have different microbial communities (66). Our results also have an implication for the application of strain mixtures, which is thought to be an effective method to enhance biocontrol activity (49, 59). Closely related strains sharing the same trait and niche may need to be avoided since competition in the rhizosphere is likely to result in the displacement of less competitive strains and compromise their potential to add to the activity of the mixture.

Finally, we tested the effect of the pea cultivar on rhizosphere colonization by indigenous and introduced 2,4-DAPG-producers. Previous studies have documented differential responses of plant cultivars to beneficial microorganisms. For example, cultivars of potato (4), maize and grass (48) that differed in their susceptibility to pathogens also differed quantitatively and qualitatively in their rhizosphere antagonistic microflora. In addition, the ability of crop monoculture to render soils suppressive to pathogens is affected by the cultivar planted, as was shown for certain watermelon cultivars that induce suppressiveness to Fusarium wilt (35), and wheat cultivars that induce suppressiveness in orchard soils to apple replant disease (43). Mazzola et al. (44) showed that the wheat cultivar Lewjain selected greater proportions of *phlD*$^+$ pseudomonads as compared to the other tested cultivars. The first report of the differential response of pea cultivars to inoculation with *P. fluorescens* strains was published by Åström and Gerhardson (2). They found that an increase in shoot dry weight by inoculated bacterial isolates varied with the pea cultivar. On the other hand,

another study showed that four pea cultivars did not differ in the populations of a *Burkholderia cepacia* strain on roots (30).

In our work, the effect of the host crop cultivar on the selection of indigenous *phlD*$^+$ fluorescent *Pseudomonas* was studied with four pea cultivars. There was considerable variability among the cultivars, with 'Little Marvel' supporting the highest and 'Quincy' the lowest populations of 2,4-DAPG producers. In the first and second cycles, differences in population sizes between these two cultivars were about 1,000- and 10-fold, respectively (Fig. 5). The effect of the pea cultivar on root colonization was then assessed by comparing the population dynamics of introduced strains Q8r1-96 and MVP1-4 on eight cultivars. Both strains efficiently colonized the rhizosphere of all eight pea cultivars; however, some interesting differences emerged. For example, although both strains are "premier" colonists of pea, MVP1-4 did not differ significantly across the eight pea cultivars; but densities of Q8r1-96 showed considerable differences in all cycles (Fig. 6). MVP1-4 was isolated from pea roots from the continuous pea field in Mount Vernon, WA (32), pointing to a better adaptation of this strain to different pea cultivars. During the more than 30 years of pea monoculture the field served as a nursery, literally hundreds of cultivars or lines were grown in the field. In contrast, strain Q8r1-96 was isolated from wheat grown in TAD soil that had undergone wheat monoculture. Our previous studies (32, 33, 55) clearly indicate that the BOX-PCR genotype of a strain is predictive of its rhizosphere competence on a given crop. However, these results suggest that long term contact with a crop may refine even further its ability to colonize and interact with that crop.

In conclusion, our results showed an important effect of the host crop genotype as a modulator of rhizosphere colonization and competitiveness of 2,4-DAPG-producing

Pseudomonas strains. To our knowledge this is the first report indicating that plant species have a pronounced effect on the outcome of the competition among rhizobacteria sharing a biocontrol trait. Depending on the bacterial strain combination in a mixture, and the host rhizosphere to which it is applied, a biocontrol strain can drastically reduce its population size therefore compromising its biocontrol activity.

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Table 1. Selection of indigenous populations of 2,4-DAPG-producing fluorescent *Pseudomonas* by different crop species

Host crop[a]	Mean colonization Log (CFU g^{-1} root)[b]	AUCPC[c]
Alfalfa	6.16	30.31 bcd[d]
Barley	5.88	27.06 e
Beans	6.86	31.14 bc
Flax	4.82	22.25 f
Lentils	7.50	35.51 a
Lupines	6.52	28.81 cde
Oats	4.99	21.55 f
Peas	7.14	32.58 b
Wheat	5.75	28.01 de

[a] Seeds were sown in a pea wilt-suppressive soil and plants were grown in a growth chamber. For details see Materials and Methods.

[b] Mean colonization was calculated as the mean population density across all cycles (32). Population data were converted to log CFU g^{-1} fresh weight of root.

[c] The area under the colonization progress curve (AUCPC) was calculated by using the trapezoidal integration method (10).

[d] Means in the same column followed by the same letter are not significantly different ($P > 0.05$) according to Fisher's protected LSD**.**

Table 2. Competition among *Pseudomonas fluorescens* Q8r1-96, F113 and MVP1-4 in the wheat and pea rhizospheres.

	AUCPC[b]					
	Wheat			Pea		
Inoculation[a]	Q8r1-96	F113	MVP1-4	Q8r1-96	F113	MVP1-4
Q8r1-96	28.10 bc[c]	-	-	22.14 c	-	-
F113	-	30.21 a	-	-	22.69 bc	-
MVP1-4	-	-	25.98 e	-	-	25.47 a
Q8r1-96+F113	27.41 bcd	29.76 a	-	21.66 c	22.50 bc	-
Q8r1-96+MVP1-4	27.97 bc	-	26.38 de	18.63 de	-	25.14 a
F113+MVP1-4	-	28.49 b	23.74 f	-	19.53 d	23.73 b
Q8r1-96+F113+MVP1-4	27.15 cde	27.63 bc	23.38 f	18.54 de	17.41 e	26.13 a

[a] A non-cropped soil was inoculated with *P. fluorescens* Q8r1-96, F113 or MVP1-4 alone or mixtures of these strains. The same inoculated soil was planted to wheat or pea and incubated in the growth chamber. For details see Materials and Methods.

[b] The area under the colonization progress curve (AUCPC) was calculated by using the trapezoidal integration method (10).

[c] Means corresponding to the same crop and followed by the same letter are not significantly different ($P > 0.05$) according to Fisher's protected LSD**.**

Figure 1. Selection of indigenous populations of 2,4-DAPG-producing *Pseudomonas* from a Fusarium wilt suppressive soil by the rhizosphere of different crops. The soil was seeded to alfalfa (▭), barley (■), bean (▭), flax (▨), lentil (▨), lupine (▭), oat (▭), pea (▨), and wheat (▨). Plants were incubated in a growth chamber, harvested after 3 weeks (one "cycle"), and the soil was replanted to new seeds during six cycles (for details see Materials and Methods). The total population of *phlD*$^+$ fluorescent *Pseudomonas* was enumerated after each cycle. Arithmetic mean values are shown on the graph. Different letters for the same cycle correspond to significant differences ($P < 0.05$)

Figure 2. Growth kinetics of *Pseudomonas fluorescens* strains in vitro at different incubation temperatures. *P. fluorescens* strains Pf-5 (●), Q2-87 (○), Q8r1-96 (▼), MVP1-6 (▽), F113 (■), 1M1-96 (□), MVP1-4 (◆) and MVW1-1 (◇) were grown on 1/3x KMB broth. Cultures were incubated at 15°C (A) and 22°C (B) and bacterial growth was measured by OD_{600nm} during 47 h. The experiment was repeated twice, and means from a representative experiment are shown.

Figure 3. Rhizosphere competitiveness among 2,4-DAPG-producing strains on wheat and pea. Natural soil was inoculated with each bacterial strain alone, 1:1 or 1:1:1 mixtures and seeded to wheat (first column) or pea (second column). Bacterial populations in the rhizosphere were measured during five consecutive cycles of 3 weeks each. In each graph, rhizosphere populations of Q8r1-96 (■, ▨, genotype D), F113 (▭, ▨, genotype K) and MVP1-4 (▭, ▥, genotype P) strains are compared in single (solid bars) and mixed inoculation treatments (dashed bars). Letters on each graph correspond to genotypes of strains used for the corresponding mixture. Populations of each strain

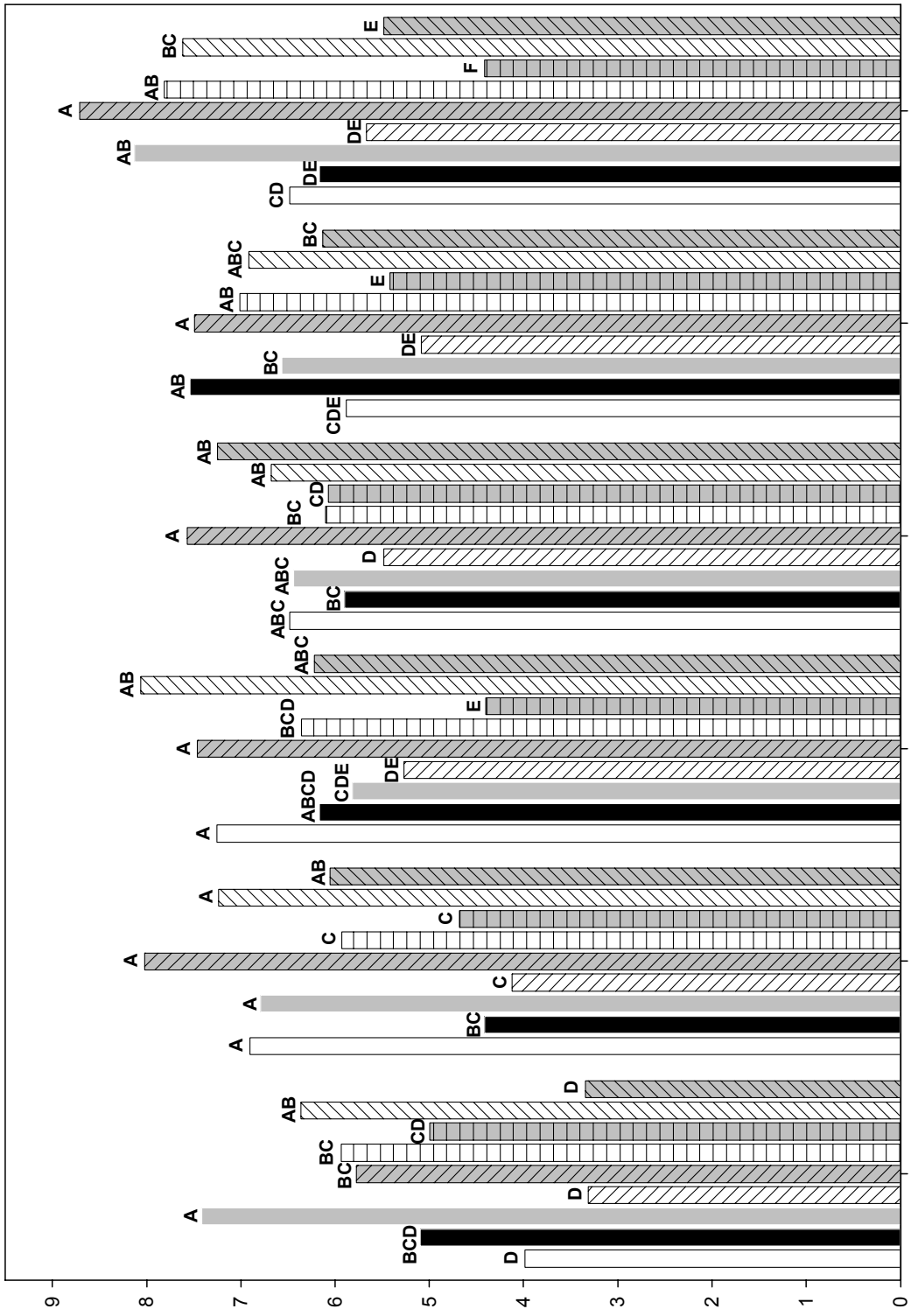
were determined by PCR by using allele-specific primers. (*) Indicates populations below detection limit (log 3.26 CFU g^{-1} of root). Population values at cycle "0" correspond to soil inoculated at the beginning of the experiment and are expressed as log CFU g^{-1} soil. The experiment was repeated three times, and arithmetic mean values from a representative experiment are shown. Different letters for the same cycle correspond to significant differences ($P < 0.05$). Rhizosphere populations of total culturable heterotrophic bacteria are shown in Fig. 4.

Figure 4. Rhizosphere populations of total culturable heterotrophic bacteria from a natural non-cropped soil. Samples corresponding to the competitiveness study on wheat and pea (Fig. 3, Table 2) were analyzed for total bacteria populations by growing on 1/10x TSB^{+}. A Shano sandy loam was inoculated with *P. fluorescens* strains Q8r1-96 (●), F113 (○), MVP1-4 (▼), Q8r1-96+F113 (▽), Q8r1-96+MVP1-4 (■), F113+MVP1-4 (□), Q8r1-96+F113+MVP1-4 (◆), or methylcellulose (non-inoculated control) (◇). Inoculated soils were sown to wheat (A) or pea (B) and grown during five consecutive cycles of 3 weeks each (see Materials and Methods for more details). The experiment was repeated three times, and arithmetic mean values from a representative experiment are shown. Population data corresponding to inoculated $phlD^{+}$ strains is shown in Fig. 3.

Figure 5. Selection of indigenous populations of fluorescent *Pseudomonas* and 2,4-DAPG-producing *Pseudomonas* from a natural soil by the rhizosphere of pea cultivars. A Fusarium wilt suppressive soil was seeded with four pea cultivars: WSU31 (■), Little marvel (▭), Columbian (▭) and Quincy (▨). Plants were incubated in a growth chamber, harvested after 3 weeks (one "cycle"), and the soil was replanted to new seeds

during two cycles. The total population of fluorescent *Pseudomonas* (A) and *phlD*$^+$ fluorescent *Pseudomonas* (B) was enumerated after each cycle. Population values are expressed as log CFU g^{-1} soil. Different letters for the same cycle correspond to significant differences ($P < 0.05$).

Figure 6. Rhizosphere colonization of pea cultivars by *P. fluorescens* Q8r1-96 and MVP1-4. A non-cropped soil was inoculated with either *P. fluorescens* Q8r1-96 (A) or MVP1-4 (B) and seeded with eight pea cultivars: WSU31 (■), Little marvel (▭), Columbian (▭), Quincy (▨), Rex (▨), Alaska (▭), Majorette (▭), and Joel (▭). Plants were incubated in a growth chamber, harvested after 3 weeks (one "cycle"), and the soil was replanted to new seeds during four cycles. The population of introduced *phlD*$^+$ strains was enumerated after each cycle. Populations values at cycle "0" correspond to soil inoculated at the beginning of the experiment and are expressed as log CFU g^{-1} soil. Different letters for the same cycle correspond to significant differences ($P < 0.05$). (*) Indicates treatments contaminated with MVP1-4.


Log (CFU g-1 fresh root weight)
Cycle
0
1
2
3
4
5
6
7
8
1
2
3
4
5
6
D
BCD
A
D
BC
BC
CD
AB
D
A
BC
C
C
A
A
AB
A
A
ABCD
CDE
DE
A
BCD
E
AB
ABC
ABC
BC
ABC
D
A
BC
CD
AB
AB
CDE
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ABC
BC
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DE
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A
DE
AB
F
BC
E

Figure 1

Figure 2

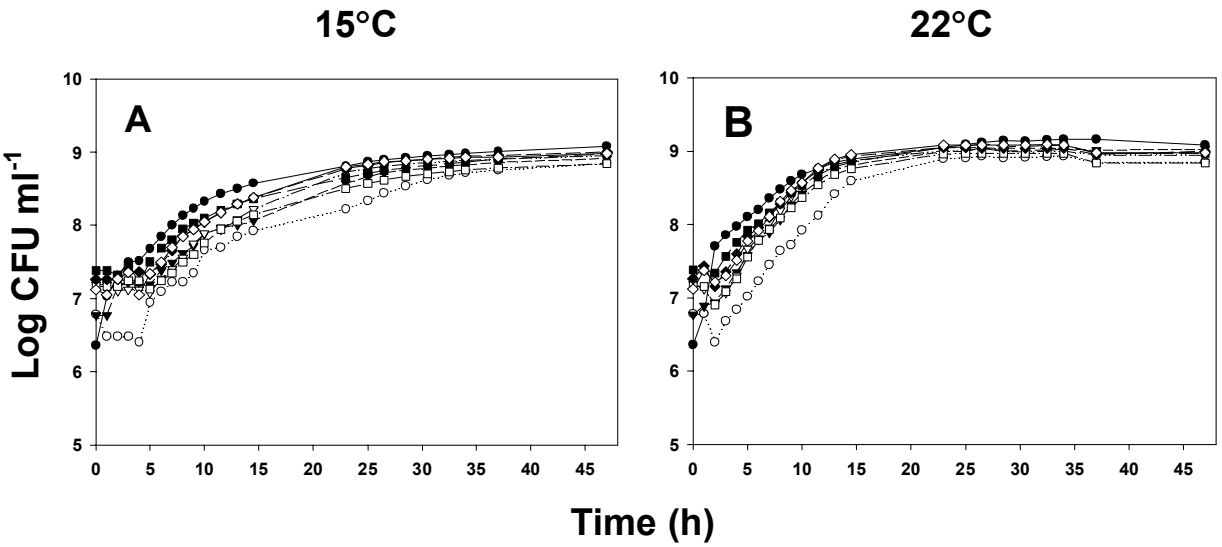


Figure 3

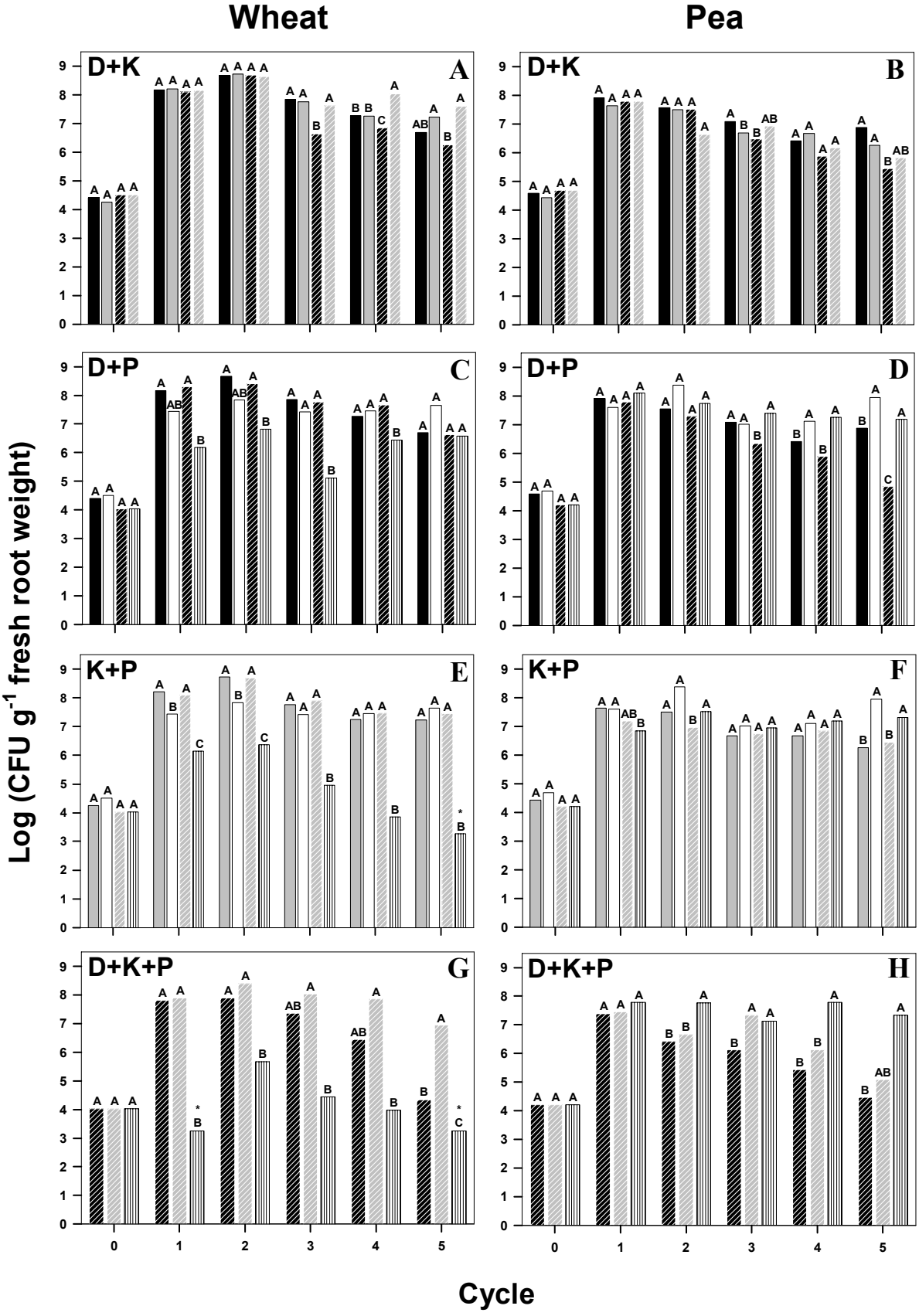

Wheat
Pea
D+K
A
B
D+P
C
D
K+P
E
F
D+K+P
G
H
Log (CFU g-1 fresh root weight)
Cycle
0
1
2
3
4
5

Figure 4

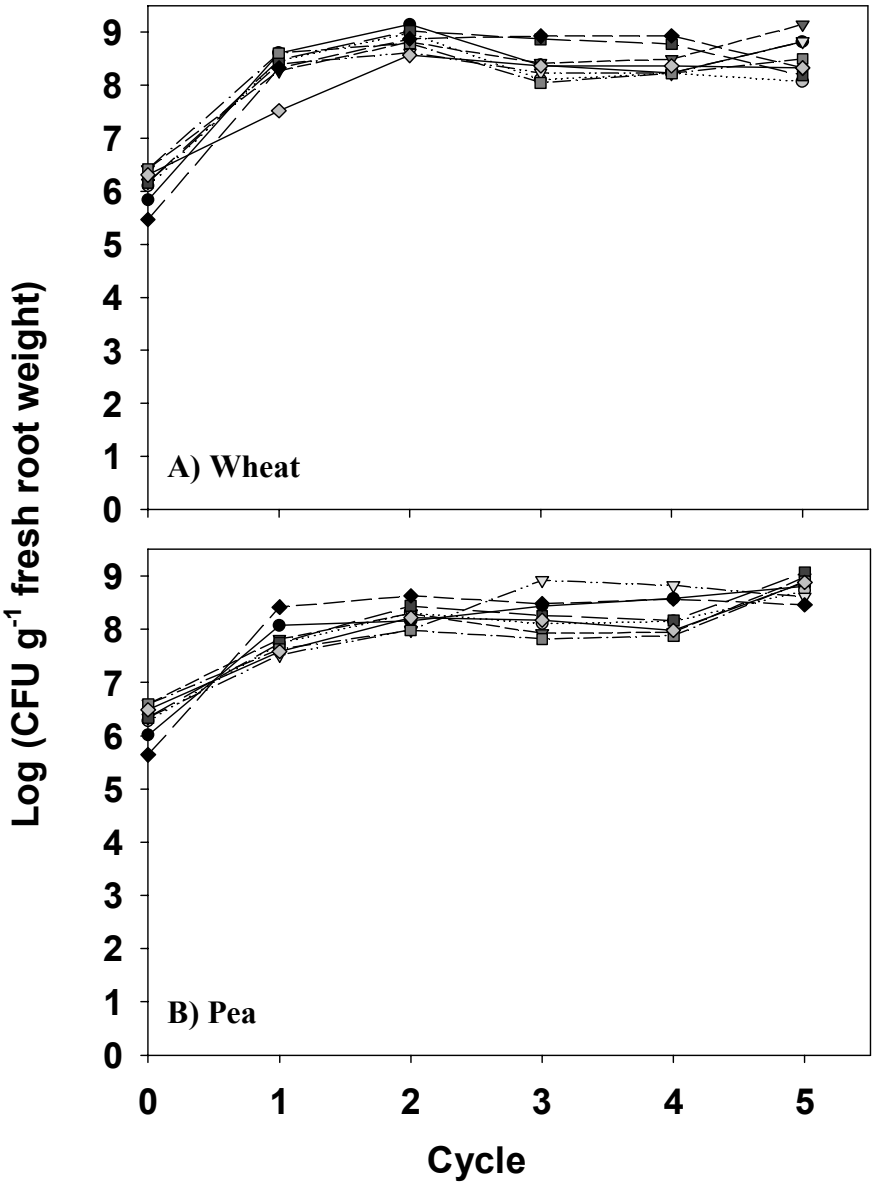


Figure 5

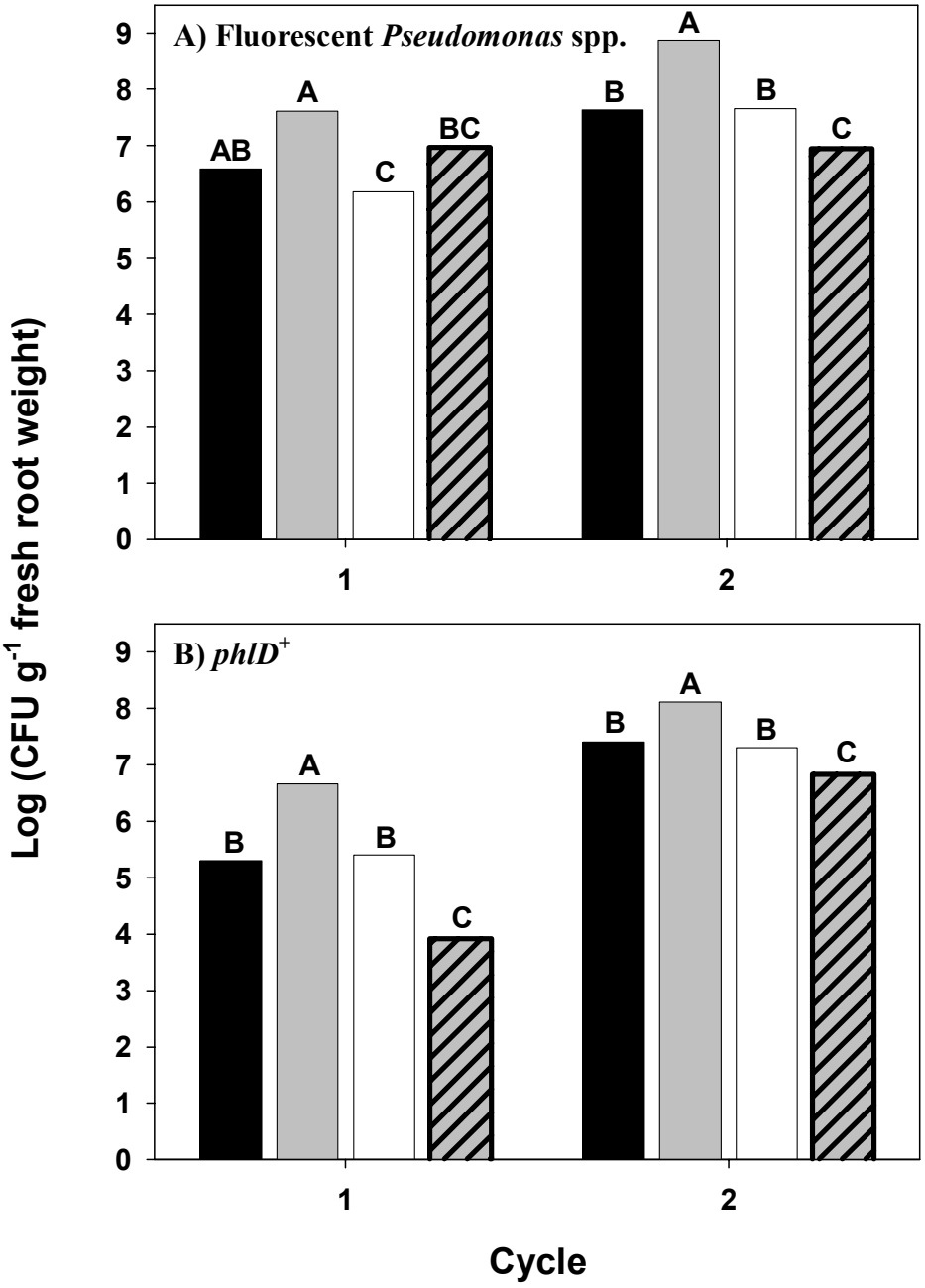
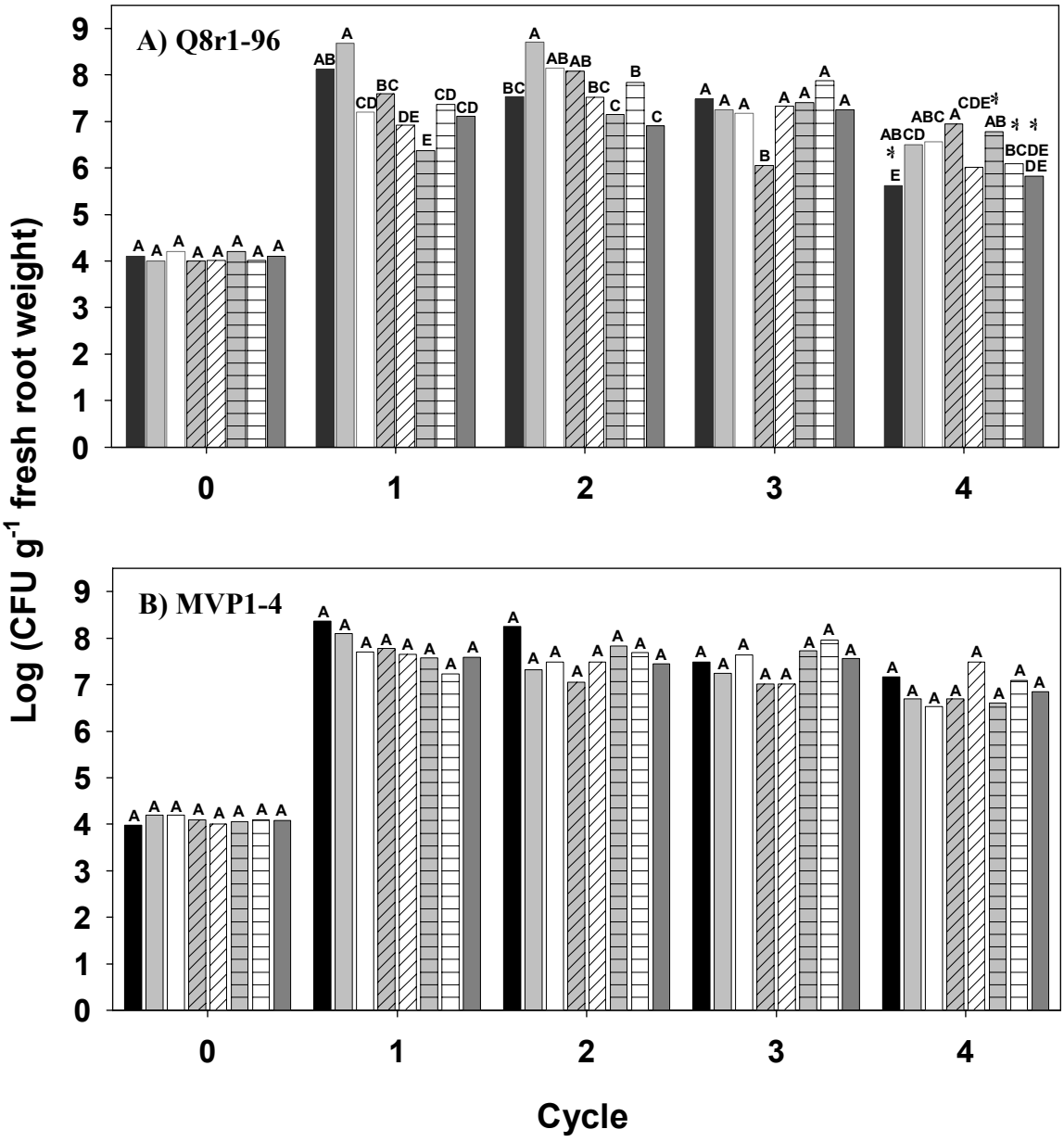

A) Fluorescent *Pseudomonas* spp.
B) *phlD*$^+$
Log (CFU g^{-1} fresh root weight)
Cycle
AB
A
C
BC
B
A
B
C
B
A
B
C
B
A
B
C
0
1
2
3
4
5
6
7
8
9

Figure 6


A) Q8r1-96
B) MVP1-4
Log (CFU g-1 fresh root weight)
Cycle
0
1
2
3
4
0
1
2
3
4
5
6
7
8
9

CHAPTER 3

Utilization of Trehalose, Benzoate, Valerate, and Seed and Root Exudates as Sole Carbon Sources is Not Correlated With Superior Rhizosphere Colonization by 2,4-diacetylphloroglucinol-Producing *Pseudomonas* spp.

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Keywords: *Pseudomonas*, rhizosphere colonization, root exudates, seed exudates, trehalose, benzoate, valerate, 2,4-diacetylphloroglucinol, biological control.

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ABSTRACT

Fluorescent *Pseudomonas* spp. producing the antibiotic 2,4-diacetylphloroglucinol (2,4-DAPG) are effective biological control agents against several soilborne pathogens. A previous study showed that superior ("premier") root colonizer *P. fluorescens* Q8r1-96 differed from two average colonizers in the utilization of trehalose, benzoate and valerate as sole carbon sources. We tested the utilization of trehalose, benzoate and valerate by a collection of 55 2,4-DAPG-producing fluorescent *Pseudomonas* spp. strains, and we found no correlation between the ability to utilize these carbon sources and rhizosphere competence. 73%, 48%, and 69% of strains were able to utilize trehalose, benzoate and valerate as sole carbon sources, respectively. We found a correlation between the utilization of these compounds and previous groupings of these strains by BOX-PCR. Twenty three strains grew efficiently on wheat and pea root and seed exudates, with doubling times between 0.9 and 1.6 h generation^{-1} and lag phases between 5 and 8 h, comparable to growth in glucose as a sole carbon source. Only 10 strains including strains with premier (Q8r1-96) and average (Q2-87) rhizosphere competence showed slower growth in wheat root exudates, with lag phases between 16 and 22 h. Results were the same when soil was added to the culture media. Growth of four strains in minimal medium containing glucose, wheat seed exudate or pea seed exudates was not influenced by whether culture media were obtained from wheat seeds or rich medium. We conclude that the superior ability of some strains to colonize the roots of certain crops can not be explained by the utilization of the carbon sources tested in our study.

INTRODUCTION

Strains of fluorescent *Pseudomonas* spp. from soil and the rhizosphere produce several metabolites active against plant pathogens (16, 56). Of special interest are strains producing the antibiotic 2,4-diacetylphloroglucinol (2,4-DAPG), which has a broad activity against fungi, oomycetes, bacteria, viruses and nematodes (4, 16), and is phytotoxic at high concentrations (20). Isolates of 2,4-DAPG-producing *Pseudomonas* spp. are effective biocontrol agents (49, 56), and naturally-occurring populations of these bacteria are involved in the suppression of soilborne pathogens in some soils (60). The biosynthetic locus for 2,4-DAPG includes six genes, *phlF* and *phlACBDE* (4). *phlD*, a key gene in the biosynthesis of the antibiotic, is well conserved in nature (8, 21, 50) and is widely used for identification of 2,4-DAPG-producing bacteria. 2,4-DAPG-producing fluorescent pseudomonads have been isolated from soils and roots worldwide (7, 21, 27), and molecular fingerprinting techniques such as BOX-PCR (37) and *phlD*-RFLP (34), have described 22 genotypes (A-T, Pf-Y, Pf-Z) (25, 26, 36, 38). Isolates from different genotypes differ in ability to colonize the rhizosphere of crops (rhizosphere competence) (24, 25). A distinction has been made between the superior ("premier") rhizosphere competence of some strains, which reach and maintain large population sizes for long periods of time in the rhizosphere (48, 60); and the more common rhizobacterial strains designated "average" colonizers, whose rhizosphere populations decline within days or weeks after inoculation and do not persist in the soil (48). Superior rhizosphere competence is a key trait required for a biocontrol agent to consistently succeed in protecting plants against soilborne pathogens. Variable colonization of strains introduced into a non-native ecosystem is recognized as a major impediment to biocontrol.

Many studies have identified a wide variety of bacterial genes and traits in *Pseudomonas* spp. that contribute to rhizosphere colonization: examples include type 4 pili and flagella, O-antigen, synthesis of amino acids and vitamins, site-specific recombinases, NADH-dehydrogenases (32), and enzymes such as does encoded by *dsbA* and *ptsP* (35). Other studies have focused on the physiological basis of root colonization (15, 31). For example, Raaijmakers and Weller (48) compared the utilization of 53 substrates by the "premier" colonizer *P. fluorescens* Q8r1-96 and two average colonizer strains Q2-87 and 1M1-96. Because trehalose, benzoate and valerate were utilized by Q8r1-96 but not by the other two strains in this study, we have investigated whether these compounds are metabolized preferentially by other 2,4-DAPG producers with superior colonizing ability.

Trehalose is a nonreducing disaccharide of glucose units which is utilized by many plants, animals, fungi and bacteria as a carbon and energy source (9). When synthesized by organisms, trehalose protects against stress conditions such as desiccation and dehydration, as demonstrated in bacteria (28, 54) and slime molds (10), mainly by its capacity to stabilize membranes (6). The operon for utilization of trehalose in *P. fluorescens* ATCC17400, consists of *treP*, *treA* and *treR*, and is induced by the presence of trehalose in the medium (33).

Bacteria from different genera, including *Rhizobium* spp., *Bradyrhizobium* spp., *Pseudomonas* spp. and *Azospirillum* spp., are attracted to simple aromatic compounds found in the soil, sediments and the rhizosphere (43). *P. putida* (17), *A. lipoferum* and *A. brasilense* (30) are attracted to benzoate. Genes responsible for the utilization of benzoate were identified in *P. aeruginosa*, and shown to be related to the genes in *P. putida* (61). Valeric or pentanoic acid is found in the perennial herb valerian (*Valeriana officinalis* L.)

and is used in perfumes and drugs. Valeric acid was found among the organic acids present in wheat root exudates (51). To our knowledge there is no information regarding a role of this compound in the relationship of plants with the associated microflora.

The rhizosphere is defined as the region in the soil surrounding the root which is under the influence of the plant and supports a greater biomass compared to the bulk soil. Plant roots are the main source of energy and nutrients for microorganisms living in the rhizosphere, because they exude carbon compounds and can disolve nutrients from the soil. Isolates of 2,4-DAPG-producing fluorescent *Pseudomonas* spp. belonging to certain genotypes preferentially colonize the rhizosphere of specific crops (24, 25), and we have hypothesized that this prefenrence might be explained by differential utilization of root exudates. The composition of root exudates includes high molecular weight polysaccharides as well as low molecular weight compounds such as sugars, simple polysaccharides, amino acids, organic acids, phenolic compounds, nucleotides and enzymes (5, 51). These compounds can act as a food source, or, in some cases, as signal molecules for the plant-associated microflora. Seed exudates are crucial for the establishment of associated bacteria that will affect plant development and health (41). The compounds released by the seed after imbibition are mainly low molecular weight sugars, amino acids, organic acids, fatty acids, and phenolic compounds (41). Rapid utilization of seed exudates can grant an advantage to some biocontrol bacteria over seed pathogens, as has been demonstrated for fluorescent pseudomonads antagonizing *Pythium ultimum* (12). Most seeds are vulnerable to seed-infecting pathogens in the first 12-24 h after germination (40), therefore an effective biocontrol agent should be able to grow and express biocontrol traits on seed exudates in that short period of time.

The objective of this study was to understand the physiological basis for the superior root colonizing ability of some strains of 2,4-DAPG-producing fluorescent *Pseudomonas* spp. We found no correlation between the utilization of trehalose, benzoate and valerate as sole carbon sources and the ability to effectively colonize roots by bacterial strains. In addition, all 2,4-DAPG-producers representing 17 BOX-PCR genotypes grew efficiently in root and seed exudates obtained from wheat and pea. Only on wheat root exudates did some strains show slower growth kinetics, and those strains included representatives of both premier and average rhizosphere colonizers.

MATERIALS AND METHODS

Bacterial strains and growth media. All fluorescent *Pseudomonas* spp. strains used in this study produce the antibiotic 2,4-DAPG and have been described previously (Table 1). *P. fluorescens* strain Phl1C2 was kindly provided by P. Lemanceau (Universite de Bourgogne, Dijon, France). Spontaneous rifampicin-resistant mutants of *P. fluorescens* strains Q2-87, Q8r1-96, F113 and MVP1-4 (24, 25) were used in the seed coating studies. All strains were routinely cultured at 28^{o}C on one-third-strength King's medium B (1/3x KMB) broth or agar (22). For studies on carbon source utilization the following media were prepared without adding the carbon source recommended for each medium: Ayers medium (1), Minimal Medium for *Pseudomonas* (MMP) (29), and the salts for M9 Minimal Medium (Difco®) plus the addition of $MgCl_2$ 0.24 mg/ml and $CaCl_2$ 0.011 mg/ml (M9). Stock cultures were stored in 1/3x KMB broth plus 20% glycerol at -80ºC. When appropriate, antibiotics were added to media at the following concentrations: ampicillin (Amp), 40 μg ml^{-1}; chloramphenicol (Cm), 13 μg ml^{-1}; cycloheximide (Chex),

100 μg ml^{-1}; Mycotoxin (Nystatin®, Nys, Sigma Chemicals, St. Louis), 50 μg ml^{-1}; and rifampicin (Rif), 100 μg ml^{-1}.

Utilization of trehalose, benzoate and valerate as sole carbon sources. Overnight cultures of bacterial strains in 1/3x KMB were harvested by centrifugation in a microfuge (10,000 x g, 5 min), suspended in MMP, and the concentration adjusted to an absorbance at 600nm (OD_{600nm}) of 0.1 by using a microplate reader (MR 500, Dynatech Laboratories, Burlington, MA). Suspensions were diluted 1:10 (approximate starting concentration of $1x10^7$ CFU ml^{-1}) and added (200 μl per well) to 96-well microtiter plates. Samples were replicated three times and most strains were tested twice. The plates were incubated at 28°C for 40-63 h (depending on the carbon source) and bacterial growth was monitored by measuring OD_{600nm} with the microplate reader. The lag phase duration and doubling times (during logarithmic growth phase) were estimated from the bacterial growth curves. Carbon sources were added to MMP as follows:

(i) **Trehalose**. In order to determine the optimal media and trehalose concentration for growth kinetic studies, a subset of bacterial strains was tested initially on Ayers or MMP medium supplemented with trehalose (Sigma Chemicals) concentrations ranging from 1 to 30 mg ml^{-1}. All subsequent experiments were carried out utilizing MMP supplemented with 2.5 mg ml^{-1} of trehalose.

Simultaneously with one of the growth kinetic experiments in broth (see above), viable cell numbers were estimated with the purpose of establishing a correlation with optical density. *P. fluorescens* strains CHA0, Q8r1-96, MVP1-6 and F113 were grown in MMP supplemented with 2.5 mg ml^{-1} of trehalose and 10-μl samples were taken at 2-h intervals between 10 and 24 h of growth. Aliquots were diluted serially and viable cells were scored by plating on 1/3x KMB agar. The correlation between OD_{600nm} and viable

cells obtained in this experiment was used in all the growth kinetic experiments of this study to calculate the CFU ml^{-1} values.

(ii) **Benzoate**. Initially a subset of bacterial strains was grown in MMP supplemented with benzoic acid (sodium salt, Sigma Chemicals) at concentrations ranging from 1 to 20 mg/ml. A concentration of 2 mg/ml of benzoic acid in MMP was used in all the subsequent growth kinetic experiments.

(iii) **Valerate**. The n-valeric acid solution (Sigma Chemicals) was adjusted to pH 7.0 with 3M NaOH prior to use. MMP supplemented with valeric acid at concentrations of 0.1% to 1.5 % (active ingredient) was used initially to check the growth of a subset of bacterial strains. All subsequent growth kinetics experiments were carried out in MMP supplemented with 0.2% of valeric acid.

Preparation of root exudates. Wheat (*Triticum aestivum* L. cv. Penawawa) and pea (*Pisum sativum* L. cv. Columbian) seeds were surface-sterilized with 70% ethanol and 1.2% sodium hypochlorite (4 min and 1 min each treatment for wheat and pea, respectively), then washed with sterile deionized water six times, and dried between sterile Whatman filter papers. Seeds were incubated on moistened filter paper in sterile Petri plates at room temperature for 3 (wheat) and 5 days (pea). Seedlings (20-30 for wheat and 13-20 for pea) were placed in a sterile 250-ml Erlenmeyer flask (6 flasks were used for each set that was repeated 3-5 times) containing 25 ml (wheat) or 30 ml (pea) of M9 medium supplemented with Cm, Amp and Nys ($M9^{+++}$), and incubated in a light room set for a 12-h photoperiod and 22°C. After 11 days, the culture solutions were transferred to conical 50-ml tubes, centrifuged (Thermo IEC, Needham Heights, MA) (9,000 x g, 5 min at 10°C) to discard plant debris, and filtered (0.22µm pore size, Millipore Carrighwohill, Co. Cork, Ireland) prior to storage at 4°C. One hundred-

microliter samples taken before and after filter sterilization were plated on Nutrient agar (NA) (Difco®) to test for microbial contamination. One set of root exudates was collected from non-sterile wheat and pea seeds. In one set of wheat root exudates, the $M9^{+++}$ medium was supplemented with natural soil microflora by adding 0.1% (w/v) of soil from a noncropped site near Quincy, Washington, USA.

Preparation of seed exudates from wheat and pea. Seeds were surface sterilized as described above. Immediately after sterilization, seeds (60 wheat or 30 pea seeds) were placed in 250-ml Erlenmeyer flasks containing 30 ml of $M9^{+++}$ broth and incubated at 28°C in the dark until the radicle had emerged (40) (40-46 h for wheat and 60-69 h for pea). Seed exudates were harvested, tested for contamination, and stored as described above for root exudates. In addition, the seeds were placed on water agar (WA) (0.8% agar) to test for viability after the experiment.

Utilization of root and seed exudates as sole carbon sources. A two-day-old bacterial colony was suspended in M9 salts solution and washed twice by centrifugation in a microfuge (10,000 x g, 5 min). The final bacterial suspension in M9 was adjusted to an OD_{600nm} of 0.01 by using a SAFIRE (Tecan Systems, Inc., San Jose, CA) microplate reader. Suspensions were diluted 1:10 ($1x10^6$ CFU ml^{-1} approximate starting concentration) in either root or seed exudates and added (200 µl per well) to 96-well microtiter plates. A control treatment in each experiment consisted of samplesdiluted in M9 containing glucose (0.4%) as the sole carbon source. Samples were replicated three times and most bacterial strains were tested twice. The plates were incubated at 27°C for 48 h in the SAFIRE microplate reader and bacterial growth was monitored at one-hour intervals by measuring the OD_{600nm}. Considering the lowest OD_{600nm} values detected with the SAFIRE microplate reader, the detection limit was set at log 5.0 CFU ml^{-1}.

Seed coating. Wheat (cv. Penawawa) seeds were surface-sterilized as described above, and a sample was placed on NA plates to test for contamination. Seeds were coated with Rif-resistant *P. fluorescens* strains Q2-87, Q8r1-96, F113 or MVP1-4 as previously described (42) to a final concentration of $1x10^7$ CFU $seed^{-1}$, and a control treatment was inoculated with a sterile 1% methylcellulose solution. Inoculated seeds were stored for 3 days at 4°C before cells were recovered from the seeds. Five grams of seeds from each bacterial treatment were suspended in 15 ml of M9 broth, shaken for 5 min in a Vortex mixer, and sonicated for 1 min in an ultrasonic cleanser. After removing seeds, the suspension was centrifuged briefly in a microfuge to remove plant debris, and the supernatant was centrifuged (8,000 x g, 10 min at 10°C) to harvest the bacterial cells. The pellet was suspended in 1.5 ml of M9 broth, washed twice by centrifugation in a microfuge (10,000 x g, 5 min), and the concentration adjusted to an OD_{600nm} of 0.1. In order to test the bacteria from the seed for purity, a sample from the bacterial suspension was streaked onto 1/3x KMB plus Rif, and seven randomly picked colonies were used as templates for PCR reactions with specific primers (8) for the detection of the BOX-PCR genotypes corresponding to the introduced bacterial strains. Growth kinetics studies were performed as described above for the utilization of exudates, in three different media: M9 plus glucose (plus Chex, Cm, Amp, and Rif), and seed exudates from wheat and pea (containing Cm, Amp, Nys, and Rif). Simultaneous treatments were performed comparing inoculation with bacteria previously grown on seeds and in 1/3x KMB broth cultures.

RESULTS

Utilization of trehalose, benzoate and valerate as sole carbon sources. A preliminary experiment was conducted in order to establish a relationship between viable cell numbers and OD_{600nm} readings. *P. fluorescens* strains CHA0, Q8r1-96, MVP1-6 and F113 showed similar correlations between viable cell counts from cultures in MMP supplemented with trehalose and values of OD_{600nm} (data not shown). A mean correlation value (1 OD_{600nm}=1.53x10^9 CFU ml^{-1}) was calculated considering the four strains and the eight time points recorded during bacterial growth, and this value was used in all of the experiments to calculate CFU ml^{-1}.

In general, all bacterial strains grew efficiently over the range of concentrations initially tested for trehalose and valerate. Only on benzoate, at concentrations above 5 mg ml^{-1}, growth inhibition was observed. A large proportion of the 55 strains was able to utilize trehalose and valerate (73% and 69%, respectively) as a sole carbon source (Table 1), while only 48% of the strains were able to utilize benzoate. Kinetics studies of a subset of strains (Figure 1) showed a clear difference between strains able or not able to utilize these carbon sources. With a few exceptions, strains using the same carbon sources did not differ greatly in lag phase duration and doubling time (Table 1). When bacteria were grown on trehalose, the lag phases generally were between 8 and 10 h, and the doubling times were mostly between 4 and 8 h $generation^{-1}$ (Table 1). For growth on benzoate, the duration of the lag phase was not recorded (but was less than 10 h), and most doubling times ranged between 5 and 10 h $generation^{-1}$ (Table 1). The lag phases on valerate were longer, mostly between 16 and 22 h, and the doubling times among strains were more variable ranging from 5.6 (strain JMP7) to 36.1 (strain MVP1-4) h $generation^{-}$

^{1} (Table 1). Interestingly all D-genotype strains had doubling times between 8 and 11 h generation^{-1}, while doubling times for P-genotype strains were about 30 h generation^{-1} (Table 1). In general, all strains classified as the same BOX-PCR genotype showed the same ability to utilize a specific carbon source (Table 1). All strains of genotypes A, D, E, F, G, H, I, J, K and M were able to utilize trehalose, as well as two out of five strains of genotype P. Strains utilizing valerate included all representatives of D, E, F, G, I, J, O and P genotypes, and the A-genotype strain Pf-5. Only strains of genotypes A, D, G, H and K, and the P-genotype strain MVP1-4 and the Q-genotype strain MVW4-2 were able to utilize benzoate.

Utilization of root and seed exudates. Bacterial strains differed in their ability to utilize wheat root exudates (Table 1, Figure 2 A). A distinction was observed between strains with lag phases shorter than 7 h ("fast growers") and those with lag phases between 16 and 22 h ("slow growers"). Ten of the 33 strains tested were classified as slow growers, including all representatives of genotypes B (*P. fluorescens* Q2-87, Q2-1 and Q1-87), C (*P. fluorescens* STAD384 and P2112), F (*P. fluorescens* JMP6) and most of the genotype D strains (*P. fluorescens* Q8r1-96, L5.1-96, MVP1-6, and MVW1-2). These results were reproducible whenever root exudates were obtained from non-sterile seeds (results shown in Figure 2 A and Table 1) or seeds inbroth to which soil had been added (data not shown). A subset of strains including both slow and fast growers was tested three or four additional times on wheat root exudates from non-sterile seeds with and without added soil, and the results were the same. Results were not reproducible when wheat root exudates were obtained from surface-sterilized seeds (data not shown). We were unable to obtain completely sterile wheat or pea seeds when seeds were surface

sterilized for preparation of root exudates. Different microorganisms were isolated from among the contaminants, but none of them inhibited *P. fluorescens* strains grown on agar plates nor where the exudates themselves inhibitory in vitro (data not shown).

No differences among strains in growth kinetics were observed when strains were grown in pea root, wheat seed or pea seed exudates. On pea root exudates all bacteria had lag phases shorter than 5 h (Table 1). The results were reproducible whenever exudates were obtained from non-sterile (data shown in Table 1 and Figure 2 C) or surface-sterilized seeds. All strains utilized seed exudates rapidly, with lag phases of approximately 5 to 8 h for both wheat and pea exudates (Table 1, Figure 2 B and D). In general, no contamination was detected when seed exudates were obtained from either wheat or pea. Doubling times were similar for all strains growing in the different exudates, with values of 0.9 to 1.6 h generation^{-1}. The parameters of lag phase duration and doubling time observed during growth on exudates did not differ from the ones observed when the strains were grown in a minimal medium supplemented with glucose (Table 1, Figure 2 E).

Effect of physiological condition of the bacterial inoculum. No differences were observed in the growth kinetics of four *P. fluorescens* strains on glucose (Figure 3 A, B), wheat seed (Figure 3 C, D) or pea (Figure 3 E, F) seed exudates, when the starting cells were obtained from broth culture or seed coatings (Figure 3). The lag phase duration was comparable for each strain grown on the same carbon source and obtained from broth or seeds, except that lag phases for F113 and MVP1-4 on pea seed exudates (Figure 3 E, F), were 4-5 h longer when inocula were obtained from seed coating. Doubling times and

maximal cell density at the stationary phase were the same for all strains growing in the same condition, despite the origin of the inoculum.

DISCUSSION

The hypothesis that the ability to utilize trehalose, benzoate and valerate as sole carbon sources might be related to the superior rhizosphere competence of 2,4-DAPG-producers belonging to certain genotypes (48) was not verified in our study. Kinetics of growth of all the strains tested on these three carbon sources showed a very slow utilization of these compounds (Table 1, Figure 1), with doubling times greater than 4 h generation^{-1} (Table 1). In most cases, all strains of the same BOX-PCR genotype shared the same profile of utilization of the three carbon sources tested (Table 1). Our results confirm a phenotypic basis for the BOX-PCR genotype classification. Most of the strains tested utilized trehalose, including representatives of genotypes known to be superior rhizosphere colonizers of either wheat (25) or pea (24) such as D-, K-and some strains of P- genotype; average colonizers such as isolates of A- and E- genotypes; and strains for which information on rhizosphere colonization is not available (F-, G-, H-, I-, J- and M-genotypes). Previously, utilization of trehalose by *Pseudomonas* spp. was found to be induced by the presence of the pathogen *Pythium debaryanum* (13). Also it was found that the mycorrhizosphere of *Lacccaria bicolor* preferentially selected *P. fluorescens* strains which utilize trehalose from the soil (11). Trehalose is an important component of fungal spores (57), fruiting bodies and vegetative cells (9). For this reason the importance of trehalose utilization in biocontrol bacteria might be more related to the relationship with the fungal pathogen than with colonization of the plant rhizosphere. On the other

hand, trehalose is produced by plants such as rice, potato, and *Arabidopsis thaliana* (39), and the presence of trehalose biosynthetic genes in many higher plants suggests that they have the potential to synthesize this compound (39). Nevertheless, trehalose is present in small amounts in plants, and it is possible that it may function more as a signal molecule than a bulk metabolite (39). In *Escherichia coli*, two systems related to the utilization and endogenous synthesis of trehalose has been described (54). The utilization system involving a periplasmic trehalase is induced by osmotic stress and not by trehalose (54). In contrast a study of the trehalose utilization operon in *Pseudomonas fluorescens* ATCC17400 showed that TreA is induced by trehalose but not by high osmolarity (33), so an osmotic shock was not used to study trehalose utilization in our *Pseudomonas* spp.

Strains representative of 5 of the 17 genotypes tested were able to utilize benzoate, including superior colonizers (D- and K-genotypes, one P-genotype strain), average colonizers (A-genotype and one Q-genotype strain) and some genotypes for which no colonization information is available (G and H). Benzoate is found in soils, sediments and the rhizosphere (30) and is a chemoattractant for *Pseudomonas putida* (17), *Azospirillum* spp. (30) and other bacteria (43). Benzoate catabolism might be more related to the bioremediation ability of some bacteria (43) but it does not seem to correlate with superior rhizosphere colonization ability.

The utilization of valerate by 2,4-DAPG-producing *Pseudomonas* spp. was the slowest, with lag phase durations between 16 and 22 h and doubling times longr than 5.6 h generation^{-1} (Table 1). Large differences were found between doubling times of D- (8-10 h generation^{-1}) and P- (30 h generation^{-1}) genotype strains, both of which are superior colonizers, supporting the conclusion that utilization of valerate is not correlated with superior rhizosphere competence. Valerate is a componenet of wheat root exudates (51),

but we found no preference for this substrate among bacterial isolates obtained from wheat. Strains utilizing valerate also included representatives of superior (D, P), and average (E, O, one A-genotype strain) rhizosphere colonizers. A previous study also failed to find a correlation between the utilization of sugars as sole carbon sources and colonization ability (31). That study showed that a *P. fluorescens* mutant strain impaired in growth on major sugars present in tomato exudates (glucose, fructose and maltose) was not impaired in its ability to colonize the tomato rhizosphere (31). Rovira (51) stated that it is unlikely that ubiquitous sugars, amino acids and organic acids alone will make a crucial effect on the plant-associated microflora, but probably either the balance between them or the production of unique compounds will have a greater effect.

Obtaining root exudates is a difficult task and in most studies, as in our case, exudates are collected in a solution culture. Therefore, care should be taken when extrapolating such results to explain what occurs in the rhizosphere (5, 19, 51). Many factors affect the amount and composition of root exudates, including the plant species, age, nutrition and root damage (51), the presence of microorganisms (14, 51), the culture solution (14, 51), temperature and light (51), and the presence of microbial products (45, 59). In our study, we were unable to obtain completely axenic seeds without compromising their viability, and contamination was detected whenever seeds were surface-sterilized to obtain root exudates. Since microorganisms are known to increase the amount of exudates up to four times (51) and they transform compounds into new soluble forms (14), we decided to use non-sterile seeds to achieve conditions closer to those in the soil. The addition of soil to the culture solution also served to supplement the system with natural microflora, as previously described (14). Unexpectedly, when root exudates were prepared from surface-sterilized seeds (not completely sterile seeds, as contaminating microorganism

were observed), the results obtained with the exudates were less reproducible than when non-sterile seeds were used and soil was added to the system. This may be explained by partial metabolic processes occurring when just a few microbial groups are present. Degradation and solubilization of most compounds likely will occur more rapidly when a richer microflora is active, giving rise to exudates of more consistent composition.

Growth of the majority of 2,4-DAPG-producing fluorescent pseudomonads strains in all wheat and pea root and seed exudates was rapid, with doubling times between 0.9 and 1.6 h generation^{-1} and lag phases between 5 to 8 h, comparable to the parameters of growth in 0.4 % glucose (Table 1). Only in wheat root exudates were differences in strains growth apparent; slow growers included representatives of average colonizers (B-genotype strains), and superior colonizers (most of the D-genotype strains) (Table 1, Figure 2), indicating no correlation between kinetics of exudate utilization and colonization ability. Similarly, Gu and Mazzola reported (15) that colonization of apples by a strain of *P. putida* was not correlated with utilization of apple root exudates as a sole carbon source. However, a correlation was detected between the utilization of major organic acids and sugars present in root exudates of the grass cv. Balmutra and rhizoremediation of naphthalene by a strain of *P. putida* (23), but proof of a relationship between utilization of root exudates and rhizosphere colonization is still lacking (15, 31). In our study we did not characterize quantitatively nor qualitatively our exudates, so we cannot explain why differences in growth were observed only in wheat root exudates. Antimicrobial metabolites are released in the root exudates of some plants (3, 5), but in our study, we did not observe inhibition of bacterial strains in vitro by wheat root exudates. Because we used bulk root exudates in these studies and exudate concentrations

may not have been optimal for the detection of an inhibition zone, we cannot yet rule out and inhibitory activity.

All strains grew rapidly in pea and wheat seed exudates (Table 1, Figure 2). Seed exudates were collected until the radicle emerged, coinciding with the last event of exudation during seed germination (41). In general, contamination with natural microflora was less severe than with root exudates, probably because incubation times were only 2-3 days, as opposed to 11 days for root exudates. Nevertheless, we found no differences in utilization of either wheat or pea seed exudates by the 2,4-DAPG-producing strains tested. Fukui et al. (12) found that fluorescent pseudomonads with shorter lag phases growing on sugar beet seeds controlled pericarp colonization by *P. ultimum* better than did strains with longer lag phases. That study characterized these "fast growers" as having doubling times of 2-3 h generation^{-1} and lag phases shorter than 4 h (12). We found that most of our strains can be classified as faster growers according to the definition of Fukui et al. (12) (Table 1), as just a few of our strains had lag phases of 8h (lower limit for the "slow growers" classified in the Fukui et al. study). These results are not surprising, since many of the 2,4-DAPG-producing strains included in our study have been identified as effective biocontrol agents of root and seedling diseases.

Even if we did not observe a clear correlation between utilization of root exudates and rhizosphere colonization by 2,4-DAPG-producing strains, it is worth noting that exudates can alter soil structure, and they contain compounds such as homoserine lactones that can inhibit signals among bacteria (59). Our study shows no correlation between exudate utilization and rhizosphere colonization, but it does not rule out a crucial role for exudates in these plant-beneficial bacteria associations.

In order to determine if our results with exudates were biased by using bacterial cultures grown in rich media, we compared these conditions to those obtained with bacteria subjected to nutrient-limited conditions. Pre-growing the strains on wheat seeds did not affect their growth on glucose or seed exudates (Figure 3). Persson et al. (44) showed that a series of physiological changes occur when *P. fluorescens* strains are grown under nutrient-limiting conditions. Gu and Mazzola (15) found that a *P. putida* strain adapted to growth in poor nutrient conditions was a better rhizosphere colonizer than one grown in rich medium, but it did not utilize wheat root exudates better. We expected faster growth of superior colonizer strains (Q8r1-96, F113, MVP1-4) as compared to an average colonizer (Q2-87) when the strains were adapted to grow on seeds, but F113 and MVP1-4 had longer lag phases. The factor(s) responsible for superior root colonization by some 2,4-DAPG-producing fluorescent pseudomonads in certain crops remains elusive.

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Table 1. Bacterial strains and isolates of 2,4-DAPG-producing fluorescent *Pseudomonas* spp. used in this study and parameters of growth on different carbon sources.

			Utilization of carbon sources[a]:										
										Root Exudates		Seed Exudates	
Strain	Reference	Genotype[b]	Glucose	Trehalose		Valerate		Benzoate		Wheat	Pea	Wheat	Pea
			LPD[c]	DT[c]	LPD	DT	LPD	DT	LPD	LPD	LPD	LPD	LPD
Pf-5	18	A	6	8.9	<8[d]	21.9	16	4.6	<10	3	1	1	2
CHA0	55	A	8	9.6	<8	-[e]	-	nc[f]	<10	6	1	5	4
UP61	2	A	8	nd[g]	nd	nd	nd	nd	nd	5	1	6	5
MVP1-3	24	A	nd	5	<8	-	-	4.6	<10	nd	nd	nd	nd
Q2-87	58	B	6	-	-	-	-	-	-	23	4	4	6
Q2-1	37	B	7	-	-	-	-	-	-	16	1	6	3
Q1-87	21	B	8	nd	nd	nd	nd	nd	nd	19	3	7	4
STAD384	24	C	6	-	-	-	-	-	-	23	3	7	2
STAD376	34	C	nd	-	-	-	-	-	-	nd	nd	nd	nd
P2112	27	C	8	nd	nd	nd	nd	nd	nd	21	1	6	6
Q8r1-96	47	D	6	4.2	8	8.4	18	5.7	<10	20	4	6	7
L5.1-96	34	D	7	3.2	8	nd	nd	nd	nd	22	3	7	7
MVP1-6	24	D	8	3.8	8	10.5	18	19.11	<10	21	4	8	6
MVW1-2	24	D	7	3.3	10	6.2	16	9.87	<10	21	4	7	7
11-18	46	D	nd	3.1	8	7.0	20	7.2	<10	nd	nd	nd	nd
FTAD1R34	34	D	9	6.7	<9	9.7	18	nc	<10	7	4	8	3
ATCC49054	53	D	nd	10.0	<9	9.6	18	9.6	<10	nd	nd	nd	nd
Q128-87	34	D	nd	4.6	<9	8.4	18	11.3	<10	nd	nd	nd	nd
5WSU2	24	D	nd	7.7	<9	8.4	18	24.5	<10	nd	nd	nd	nd
MVP4-54	24	D	nd	12.0	<9	10.8	18	nc	<10	nd	nd	nd	nd
4MA30	24	D	nd	6.7	<9	7.5	16	7.5	<10	nd	nd	nd	nd

MVW2-2	24	D	nd	9.26	<9	7.7	18	nc	<10	nd	nd	nd	nd
Q8V8	34	D	nd	10.0	<9	7.3	22	nc	<10	nd	nd	nd	nd
Q8r10-96	34	D	nd	8.6	<9	9.3	16	nc	<10	nd	nd	nd	nd
2RP17	24	D	nd	7.7	<9	8.2	25	nc	<10	nd	nd	nd	nd
1WSU3	24	D	nd	4.3	<9	10.8	20	10.0	<10	nd	nd	nd	nd
OC4-1	34	D	nd	5.5	<9	10.9	22	5.4	<10	nd	nd	nd	nd
FFL1R9	34	D	nd	4.9	<9	10.6	27	nc	<10	nd	nd	nd	nd
Q2-5	34	D	nd	7.5	<9	7.5	22	8.6	<10	nd	nd	nd	nd
QT1-5	34	D	nd	3.8	<9	8.3	20	14.6	<10	nd	nd	nd	nd
Q2-2	37	E	nd	7.1	<9	8.6	38	-	-	nd	nd	nd	nd
Q37-87	21	E	nd	-	-	8.6	25	-	-	nd	nd	nd	nd
5MR2	24	E	8	4.9	8	10.9	33	-	-	6	3	8	7
QT1-6	21	E	6	4.5	10	8.1	33	-	-	7	4	7	7
JMP6	37	F	7	5.0	<9	6.0	22	-	-	16	3	7	5
JMP7	37	F	nd	8.0	36	5.6	25	-	-	nd	nd	nd	nd
FFL1R18	37	G	8	6.7	<9	7.0	16	11.6	<10	6	4	5	8
CV1-1	37	H	7	7.5	<9	-	-	10.0	<10	6	5	7	5
FTAD1R36	37	I	8	4.2	<8	8.2	16	-	-	6	2	4	8
FFL1R22	37	J	8	4.8	<9	8.5	16	-	-	6	3	4	4
FFL1R8	37	J	nd	4.0	<8	15.0	18	-	-	nd	nd	nd	nd
F113	52	K	6	9.5	12	-	-	-	-	4	2	5	3
1M1-96	48	L	5	-	-	-	-	-	-	6	5	3	6
W4-4	37	L	8	-	-	-	-	-	-	7	5	6	7
1MA1	24	L	9	-	-	-	-	-	-	5	5	5	6
Phl1C2	-	M	8	3.8	10	-	-	-	-	7	4	5	7
PILH1	21	M	6	5.4	<9	-	-	-	-	6	4	5	7
D27B1	37	M	nd	6.0	<9	-	-	-	-	nd	nd	nd	nd
HT5-1	37	N	8	-	-	-	-	-	-	6	4	6	7
7MA12	24	O	8	-	-	26.8	43	-	-	5	4	3	4
7MA20	24	O	nd	-	-	20.85	38	-	-	nd	nd	nd	nd
MVP1-4	24	P	6	7.5	<9	36.1	7	nc	<10	4	3	4	7

MVW1-1	24	P	8	10.0	<9	33.9	40	-	-	5	5	6	6
7MA15	24	P	7	-	-	26.1	16	-	-	7	5	6	7
6WSU4	24	P	nd	-	-	35.1	22	-	-	nd	nd	nd	nd
4MA6	24	P	nd	-	-	33.9	20	-	-	nd	nd	nd	nd
MVW4-2	24	Q	8	20	<9	-	-	nc	<10	5	3	4	7
MVW4-3	24	Q	8	-	-	-	-	-	-	6	5	5	4

[a] Growth kinetics of bacterial strains were assessed with minimal media supplemented with different carbon sources (for details see Materials and Methods).

[b] Genotype as determined previously by BOX-PCR (24, 37) and *phlD*-RFLP analysis (24, 34, 37).

[C] LPD = lag phase duration (h); DT = doubling time (h generation^{-1}). Values of DT for growth on glucose, root and seed exudates were similar among strains during each experiment and were approximately between 0.9 and 1.6 h generation^{-1}.

[d] Whenever the lag phase duration was not recorded, values of LPD were estimated as shorter than the first time points corresponding to logarithmic phase growth observed during the experiment.

[e] (-) = no bacterial growth was observed in minimal media supplemented with the corresponding carbon source.

[f] DT was non-calculated (nc) whenever not enough time points were recorded during the logarithmic growth phase of the bacteria.

[g] nd = non-determined.

Figure 1. Utilization of trehalose, benzoate and valerate as sole carbon sources by *Pseudomonas fluorescens* strains. *P. fluorescens* Pf-5 (●), MVP1-3 (○), Q2-87 (▼), Q8r1-96 (▽), MVW1-2 (■), F113 (□), 1MA1 (◆), MVP1-4 (◇), 7MA15 (▲), and MVW4-2 (△) were grown on MMP supplemented with either trehalose (A), benzoate (B) or valerate (C) as sole carbon sources. Cultures were incubated at 28°C and bacterial growth was measured by OD_{600nm} during 40 h (A, B) or 63 h (C). Most of the bacterial strains were tested twice on each carbon source, and means from a representative experiment are shown.

Figure 2. Utilization of root and seed exudates as carbon sources by *Pseudomonas fluorescens* strains. *P. fluorescens* strains Pf-5 (●), Q2-87 (○), Q2-1 (▼), STAD384 (▽), Q8r1-96 (■), L5.1-96 (□), MVW1-2 (◆), JMP6 (◇), F113 (▲), 1M1-96 (△), MVP1-4 (●), and 7MA15 (○) were grown on root (A, C) or seed (B, D) exudates obtained from wheat (A, B) or pea (C, D). Growth kinetics on M9 supplemented with glucose (E) served as a control. Cultures were incubated at 28°C and bacterial growth was measured by OD_{600nm} during 48 h. Most of the bacterial strains were tested twice on each exudate, and means from a representative experiment are shown.

Figure 3. Influence of the initial physiological condition of bacteria on utilization of carbon sources. Preceding the experiment, *P. fluorescens* strains Q2-87 (●), Q8r1-96 (○), F113 (▼), and MVP1-4 (▽) were grown in 1/3x KMB broth culture (first column) or on wheat seeds (second column). The bacteria were washed and grown in M9 supplemented with glucose (A, B), or seed exudates obtained from wheat (C, D) or pea (E, F). Cultures

were incubated at 28°C and bacterial growth was measured by OD_{600nm} during 48 h. Mean values from the experiment are shown on the graphs.

Figure 1

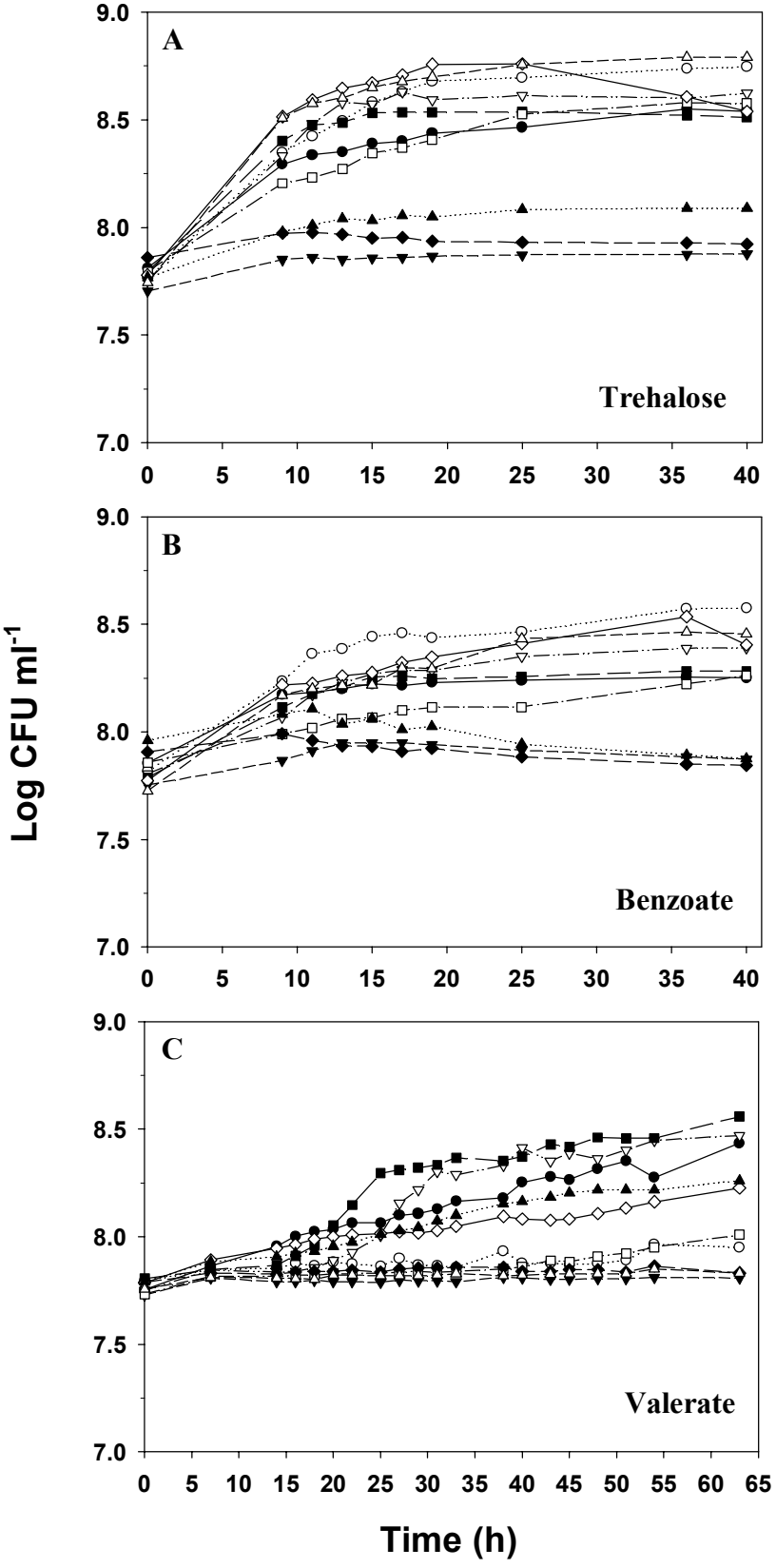

A
Trehalose
B
Benzoate
C
Valerate
Log CFU ml-1
Time (h)
9.0
8.5
8.0
7.5
7.0
0 5 10 15 20 25 30 35 40
0 5 10 15 20 25 30 35 40 45 50 55 60 65

Figure 2

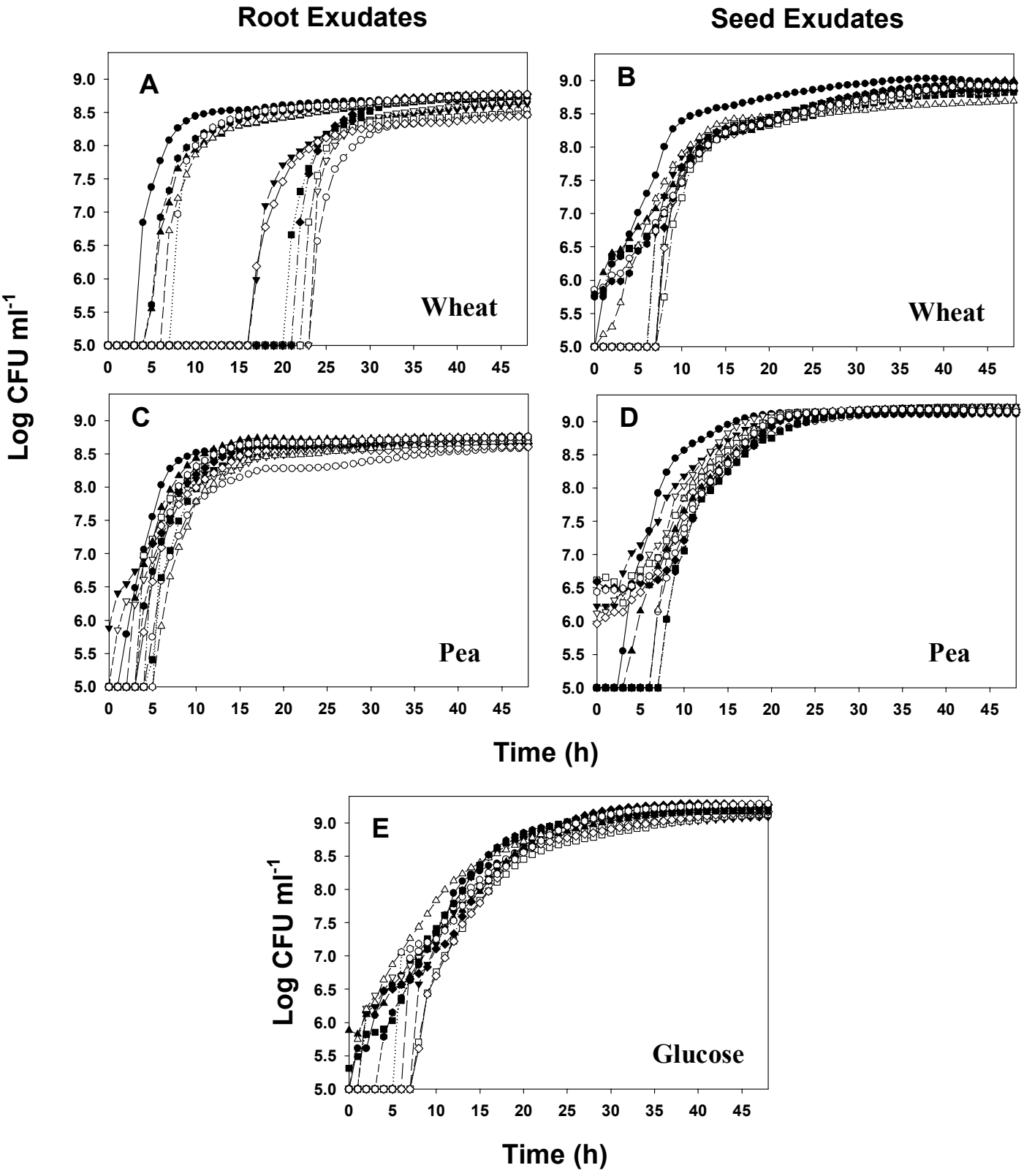
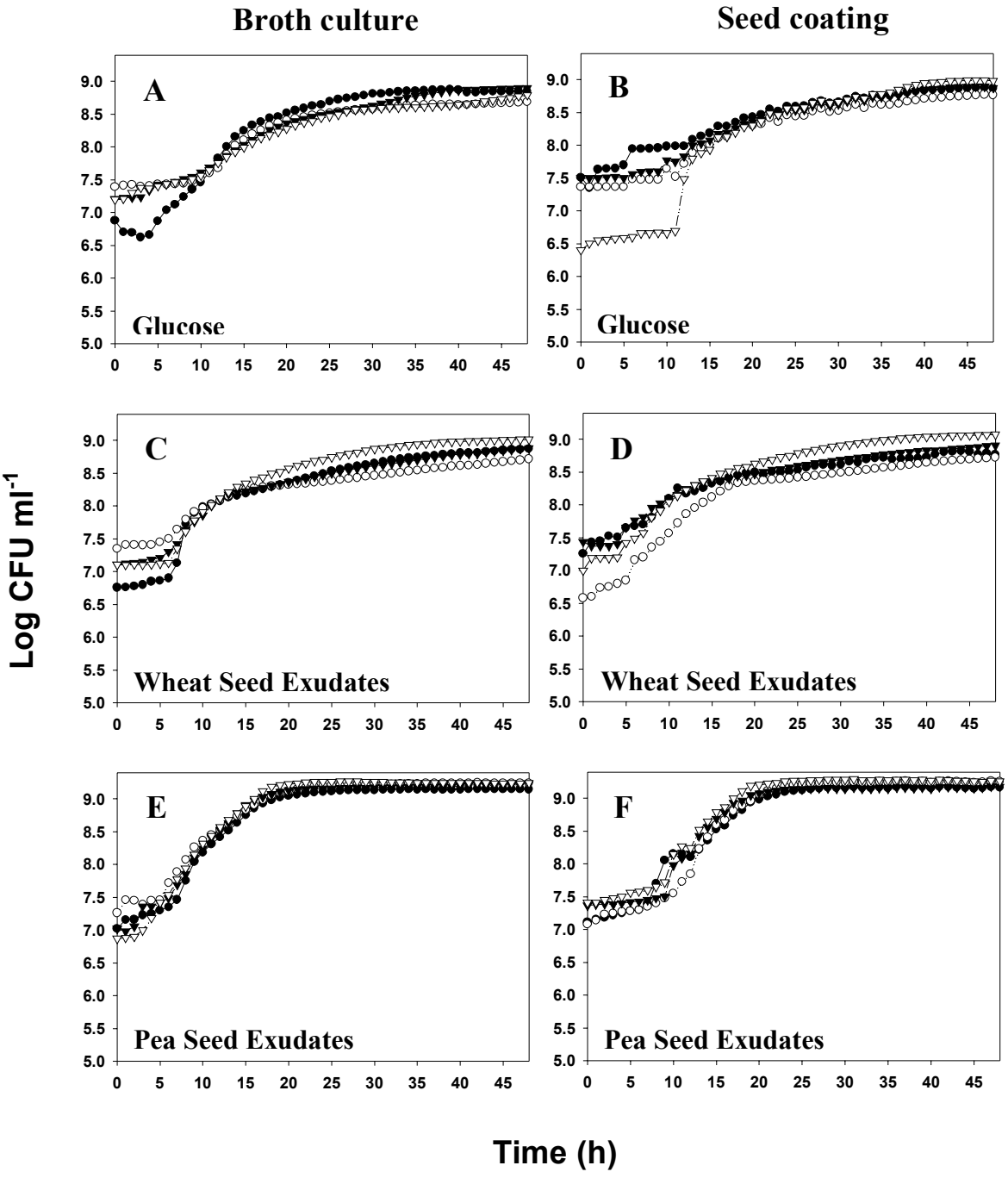

Root Exudates
Seed Exudates
A
B
C
D
E
Wheat
Wheat
Pea
Pea
Glucose
Log CFU ml-1
Time (h)

Figure 3


Broth culture
Seed coating
A
B
C
D
E
F
Glucose
Glucose
Wheat Seed Exudates
Wheat Seed Exudates
Pea Seed Exudates
Pea Seed Exudates
Log CFU ml-1
Time (h)

CONCLUSIONS

Phylogenetic analyses based on *phlD*-sequences of a worldwide collection of 2,4-DAPG-producing fluorescent *Pseudomonas* confirmed the previous genotype classification defined by BOX-PCR. Allele-specific PCR primers based on *phlD*-sequence polymorphisms were designed for the specific detection of strains of A-, B-, D-, K-, L-, and P-genotypes. These primers, used in combination with a PCR-based dilution endpoint technique, allowed detection of strains competing in the rhizosphere. A limitation of this technique was observed when antagonism occurs between competing strains in broth culture.

The host crop plays a key role in the selection of indigenous populations of 2,4-DAPG producers from the soil and in modulation of the competitiveness among introduced strains. The population sizes of indigenous 2,4-DAPG producers differed significantly in nine plant species and four pea cultivars. Moreover, an effect of the pea cultivar on rhizosphere colonization by inoculated strains was observed for Q8r1-96 but not for MVP1-4. *P. fluorescens* Q8r1-96, F113 and MVP1-4 were equally effective at colonizing the wheat and pea rhizospheres when inoculated alone into the soil, but when introduced together their rhizosphere competence changed dramatically.

We could not explain the superior ability of 2,4-DAPG producer strains to colonize the rhizosphere of certain crops by the utilization of trehalose, benzoate or valerate as sole carbon sources. A correlation was found between the utilization of these compounds and the classification of strains in BOX-PCR genotypes. The utilization of seed and root exudates from wheat and pea did not explain the superior rhizosphere colonization ability of some strains for the rhizosphere of these crops.